



**Europäisches  
Patentamt**

**European  
Patent Office**

**Office européen  
des brevets**

REC'D 09 JUL 2003

WIPO

PCT

**Bescheinigung**

**Certificate**

**Attestation**

Die angehefteten Unterla-  
gen stimmen mit der  
ursprünglich eingereichten  
Fassung der auf dem näch-  
sten Blatt bezeichneten  
europäischen Patentanmel-  
dung überein.

The attached documents  
are exact copies of the  
European patent application  
described on the following  
page, as originally filed.

Les documents fixés à  
cette attestation sont  
conformes à la version  
initialement déposée de  
la demande de brevet  
européen spécifiée à la  
page suivante.

**Patentanmeldung Nr. Patent application No. Demande de brevet n°**

02100645.7

**PRIORITY  
DOCUMENT**

SUBMITTED OR TRANSMITTED IN  
COMPLIANCE WITH RULE 17.1(a) OR (b)

Der Präsident des Europäischen Patentamts;  
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets  
p.o.

**R C van Dijk**



Anmeldung Nr:  
Application no.: 02100645.7  
Demande no:

Anmeldetag:  
Date of filing: 03.06.02  
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

DSM N.V.  
Het Overloon 1  
6411 TE Heerlen  
PAYS-BAS

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:  
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.  
If no title is shown please refer to the description.  
Si aucun titre n'est indiqué se référer à la description.)

NOVEL PECTINASE PEC60 AND USES THEREOF

In Anspruch genommene Priorität(en) / Priority(ies) claimed /Priorité(s)  
revendiquée(s)  
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/  
Classification internationale des brevets:

C12N9/00

Am Anmeldetag benannte Vertragstaaten/Contracting states designated at date of  
filing/Etats contractants désignées lors du dépôt:

AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU MC NL PT SE TR

## NOVEL PECTINASE PEC60 AND USES THEREOF

**Field of the invention**

5                   The invention relates to newly identified polynucleotide sequences comprising genes that encode novel pectinases isolated from *Aspergillus niger*. The invention features the full length nucleotide sequence of the novel gene, the cDNA sequence comprising the full length coding sequence of the novel pectinase as well as the amino acid sequence of the full-length  
10 functional protein and functional equivalents thereof. The invention also relates to methods of using these enzymes in industrial processes and methods of diagnosing fungal infections. Also included in the invention are cells transformed with a polynucleotide according to the invention and cells wherein a pectinase according to the invention is genetically modified to enhance or reduce its activity  
15 and/or level of expression.

**Background of the invention**

20                   Pectin polymers are important constituents of plant primary cell walls. They are composed of chains of 1,4-linked alpha-D-galacturonic acid and methylated derivatives thereof. Enzymes that are able to degrade the above-defined pectin polymers are called pectinases. Degradation in this respect means that at least one sugar residue or ester group has been removed from the pectin molecule. Pectinases such as polygalacturonase, pectin methylesterase, pectin  
25 lyase or pectate lyase are important for the food industry, primarily in the art of fruit and vegetable processing such as fruit juice production or wine making, where their ability to catalyse the degradation of the backbone of the pectin polymer is utilised. Another application of pectinases, in particular pectin methyl esterase, is the firming of fruit and vegetables.

30                   The seeds of leguminosae like soy beans and other types of peas also contain pectin. This pectin, however, is quite different from pectins described for fruits like apple berries etc. A large part of soybean pectin is water-insoluble. In addition soybean pectin consists mainly of xylogalacturonan and rhamnogalacturonan and contains only minor amounts of homogalacturonan. The  
35 CDTA-soluble pectic substances from soybean meal are composed of

rhamnogalacturonan and xylogalacturonan but not homogalacturonan. Due to these structural differences soybean pectin is much more difficult to degrade by pectinases as compared to fruit pectin. Even the use of technical multi-enzyme preparations was not sufficient to degrade soybean pectin structures (Huisman, MMH; Schols, HA; Voragen, AGJ, **1999**, Enzymatic degradation of cell wall polysaccharides from soybean meal, Carbohydrate Polymers 38, 299-307, and: 5 Ouhida, I; Pérez, JF; Gasa, J; **2002**, Soybean (glycine max) cell wall composition and availability to feed enzymes, J. Agric. Food Chem 50, 1933-1938).

10 An assortment of different pectin degrading enzymes is known to be present in various microorganisms such as *Aspergillus niger*.

The following is a non-exhaustive list of pectin-acting enzymes:

Enzyme	E.C. Number
exopolygalacturonase	3.2.1.67
endopolygalacturonase	3.2.1.15
pectin lyase	4.2.2.10
pectate lyase	4.2.2.2
rhamnogalacturonan hydrolase	3.2.1.x
rhamnogalacturonan lyase	4.2.2.x
rhamnogalacturonan acetylcetase	3.1.1.x
RG rhamnohydrolase	3.2.1.x
RG galacturonohydrolase	3.2.1.x
xylogalacturonan hydrolase	3.2.1.x
pectin acetylcetase	3.1.1.x
pectin methylesterase	3.1.1.11
endo-arabinase	3.2.1.99
beta-arabinofuranosidase	3.2.1.55
beta-1,4-galactanase	3.2.1.89
beta-1,3-galactanase	3.2.1.90
beta-galactosidase	3.2.1.23
alpha-galactosidase	3.2.1.22

feruloyl acetyl esterase	3.1.1.x
alpha-fucosidase	3.2.1.51
(beta-fucosidase)	3.2.1.38
beta-apiosidase	3.2.1.x
alpha-rhamnosidase	3.2.1.40
beta-rhamnosidase	3.2.1.43
alpha-arabinopyranosidase	3.2.1.x
beta-glucuronidase	3.2.1.31
(alpha-glucuronidase)	3.2.1.139
beta-xylosidase	3.2.1.37
(alpha-xylosidase)	3.2.1.x

Pectin methylesterase catalyses the removal of methanol from pectin, resulting in the formation of pectic acid (polygalacturonic acid). Pectate lyase cleaves glycosidic bonds in polygalacturonic acid by beta-elimination, pectin  
5 lyase cleaves the glycosidic bonds of highly methylated pectins by beta-elimination, and polygalacturonase hydrolyses the glycosidic linkages in the polygalacturonic acid chain.

Commercially available pectinases are actually a mixture of enzymes, which, along with other enzymes such as cellulases, are used in the  
10 fruit industry to help extract, clarify and modify fruit juices.

It is a disadvantage of these mixtures that they hardly ever contain the optimal mix of enzymes to treat a particular fruit or juice. Optimal pectin degradation depends on many factors, including the kind of fruit to be treated, the season in which the fruit is harvested, the ripeness of the fruit, and  
15 many more. A well-known example is the release of unwanted methanol and methylated pectin by the action of pectinmethylesterases and/or exo-polygalacturonases when present in too high concentrations. It is therefore an object of the present invention to provide isolated (recombinant) pectinases. The availability of isolated enzymes allows a cocktail of enzymes to be prepared  
20 which is optimized for the digestion of pectin from a particular fruit or juice.

Pectinases have been cloned in a variety of different microorganisms. Molecular cloning of pectinases in fungi has also been described. The DNA and deduced amino acid sequences of pectinases from *Aspergillus oryzae*, *Aspergillus kawachii* and *Emmericella nidulans* are known.

25 However, there is still a need for other pectinases with different

properties so that fruit processing may be optimized.

One of the disadvantages of the currently available enzymes is that they have pH optima above pH 4.0. Many fruit juices are more acidic and a pH optimum below 3.8 would be desirable. Fruit juices may become as acidic as pH 2.0 and it is therefore an object of the present invention to provide pectinases with a more acidic pH optimum. Pectinases with a pH optimum below 3.8 would be especially advantageous.

Fruit processing is also often performed at more extreme temperatures. Use of pectinases with lower temperature optima would improve yield and quality of the juice because more aromas would be released. The currently available enzymes have a very low efficiency at temperatures below 15 °C. Also, at high temperatures the currently available enzymes are easily inactivated. This is a disadvantage for those fruit juices that have to be pasteurized.

The enzymes according to the invention provide a better temperature stability and can withstand more extreme temperatures in comparison with pectinases according to the prior art.

Also, the specificity of current enzymes leaves to be desired. Fruit juice that needs to be filtrated after or during processing tends to clot the filters after some time. This is due to the inability of certain enzymes to properly digest fruit polysaccharides which causes the fouling of the filters.

The present invention addresses at least one if not all of the above problems.

#### **Object of the invention**

It is an object of the invention to provide novel polynucleotides encoding novel pectinases with improved properties. A further object is to provide naturally and recombinantly produced pectinases as well as recombinant strains producing these. Also fusion polypeptides are part of the invention as well as methods of making and using the polynucleotides and polypeptides according to the invention.

#### **Summary of the invention**

The invention provides for novel polynucleotides encoding novel pectinases.

More in particular, the invention provides for polynucleotides having a nucleotide sequence that hybridises preferably under highly stringent

conditions to a sequence according to SEQ ID NO: 1 or SEQ ID NO: 2.

Consequently, the invention provides nucleic acids that are more than 40% such as about 60%, preferably 65%, more preferably 70%, even more preferably 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% homologous to the sequences

5 according to SEQ ID NO: 1 or SEQ ID NO: 2.

In a more preferred embodiment the invention provides for such an isolated polynucleotide obtainable from a filamentous fungus, in particular *A. niger* is preferred.

10 In one embodiment, the invention provides for an isolated polynucleotide comprising a nucleic acid sequence encoding a polypeptide with an amino acid sequence as shown in SEQ ID NO: 3 or functional equivalents thereof.

15 In a further preferred embodiment, the invention provides an isolated polynucleotide encoding at least one functional domain of a polypeptide according to SEQ ID NO: 3 or functional equivalents thereof.

20 In a preferred embodiment the invention provides an pectinase gene according to SEQ ID NO: 1. In another aspect the invention provides a polynucleotide, preferably a cDNA encoding an *A. niger* pectinase whose amino acid sequence is shown in SEQ ID NO: 3 or variants or fragments of that polypeptide. In a preferred embodiment the cDNA has a sequence according to SEQ ID NO: 2 or functional equivalents thereof.

25 In an even further preferred embodiment, the invention provides for a polynucleotide comprising the coding sequence of the polynucleotides according to the invention, preferred is the polynucleotide sequence of SEQ ID NO: 2.

The invention also relates to vectors comprising a polynucleotide sequence according to the invention and primers, probes and fragments that may be used to amplify or detect the DNA according to the invention.

30 In a further preferred embodiment, a vector is provided wherein the polynucleotide sequence according to the invention is functionally linked with regulatory sequences suitable for expression of the encoded amino acid sequence in a suitable host cell, such as *A. niger* or *A. oryzae*. The invention also provides methods for preparing polynucleotides and vectors according to the  
35 invention.

The invention also relates to recombinantly produced host cells

that contain heterologous or homologous polynucleotides according to the invention.

In another embodiment, the invention provides recombinant host cells wherein the expression of an pectinase according to the invention is  
5 significantly increased or wherein the activity of the pectinase is increased.

In another embodiment the invention provides for a recombinantly produced host cell that contains heterologous or homologous DNA according to the invention and wherein the cell is capable of producing a functional pectinase according to the invention, preferably a cell capable of over-  
10 expressing the pectinase according to the invention, for example an *Aspergillus* strain comprising an increased copy number of a gene or cDNA according to the invention.

In yet another aspect of the invention, a purified polypeptide is provided. The polypeptides according to the invention include the polypeptides encoded by the polynucleotides according to the invention. Especially preferred is  
15 a polypeptide according to SEQ ID NO: 3 or functional equivalents thereof.

Fusion proteins comprising a polypeptide according to the invention are also within the scope of the invention. The invention also provides methods of making the polypeptides according to the invention.

20 The invention also relates to the use of the pectinase according to the invention in any industrial process as described herein

### **Detailed description of the invention**

#### **25 Polynucleotides**

The present invention provides polynucleotides encoding an pectinase, tentatively called PEC60, having an amino acid sequence according to SEQ ID NO: 3 or functional equivalents thereof. The sequence of the gene encoding PEC60 was determined by sequencing a genomic clone obtained from  
30 *Aspergillus niger*. The invention provides polynucleotide sequences comprising the gene encoding the PEC60 pectinase as well as its complete cDNA sequence and its coding sequence. Accordingly, the invention relates to an isolated polynucleotide comprising the nucleotide sequence according to SEQ ID NO: 1 or SEQ ID NO: 2 or functional equivalents thereof.

35 More in particular, the invention relates to an isolated polynucleotide hybridisable under stringent conditions, preferably under highly stringent conditions, to a polynucleotide according to SEQ ID NO: 1 or SEQ ID



NO: 2. Advantageously, such polynucleotides may be obtained from filamentous fungi, in particular from *Aspergillus niger*. More specifically, the invention relates to an isolated polynucleotide having a nucleotide sequence according to SEQ ID NO: 1 or SEQ ID NO: 2.

5                   The invention also relates to an isolated polynucleotide encoding at least one functional domain of a polypeptide according to SEQ ID NO: 3 or functional equivalents thereof.

10                   As used herein, the terms "gene" and "recombinant gene" refer to nucleic acid molecules which may be isolated from chromosomal DNA, which include an open reading frame encoding a protein, e.g. an *A. niger* pectinase. A gene may include coding sequences, non-coding sequences, introns and regulatory sequences. Moreover, a gene refers to an isolated nucleic acid molecule as defined herein.

15                   A nucleic acid molecule of the present invention, such as a nucleic acid molecule having the nucleotide sequence of SEQ ID NO: 1 or SEQ ID NO: 2 or a functional equivalent thereof, can be isolated using standard molecular biology techniques and the sequence information provided herein. For example, using all or portion of the nucleic acid sequence of SEQ ID NO: 1 or the nucleotide sequence of SEQ ID NO: 2 as a hybridization probe, nucleic acid molecules  
20                   according to the invention can be isolated using standard hybridization and cloning techniques (e. g., as described in Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual*. 2nd, ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989).

25                   Moreover, a nucleic acid molecule encompassing all or a portion of SEQ ID NO: 1 or SEQ ID NO: 2 can be isolated by the polymerase chain reaction (PCR) using synthetic oligonucleotide primers designed based upon the sequence information contained in SEQ ID NO:1 or SEQ ID NO: 2.

30                   A nucleic acid of the invention can be amplified using cDNA, mRNA or alternatively, genomic DNA, as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis.

35                   Furthermore, oligonucleotides corresponding to or hybridisable to nucleotide sequences according to the invention can be prepared by standard synthetic techniques, e. g., using an automated DNA synthesizer.

                  In a preferred embodiment, an isolated nucleic acid molecule of

the invention comprises the nucleotide sequence shown in SEQ ID NO: 2. The sequence of SEQ ID NO: 2 corresponds to the coding region of the A. niger PEC60 cDNA. This cDNA comprises sequences encoding the A. niger PEC60 polypeptide according to SEQ ID NO: 3.

5 In another preferred embodiment, an isolated nucleic acid molecule of the invention comprises a nucleic acid molecule which is a complement of the nucleotide sequence shown in SEQ ID NO:1 or SEQ ID NO: 2 or a functional equivalent of these nucleotide sequences.

10 A nucleic acid molecule which is complementary to another nucleotide sequence is one which is sufficiently complementary to the other nucleotide sequence such that it can hybridize to the other nucleotide sequence thereby forming a stable duplex.

One aspect of the invention pertains to isolated nucleic acid molecules that encode a polypeptide of the invention or a functional equivalent thereof such as a biologically active fragment or domain, as well as nucleic acid molecules sufficient for use as hybridisation probes to identify nucleic acid molecules encoding a polypeptide of the invention and fragments of such nucleic acid molecules suitable for use as PCR primers for the amplification or mutation of nucleic acid molecules.

20 An "isolated polynucleotide" or "isolated nucleic acid" is a DNA or RNA that is not immediately contiguous with both of the coding sequences with which it is immediately contiguous (one on the 5' end and one on the 3' end) in the naturally occurring genome of the organism from which it is derived. Thus, in one embodiment, an isolated nucleic acid includes some or all of the 5' non-coding  
25 (e.g., promotor) sequences that are immediately contiguous to the coding sequence. The term therefore includes, for example, a recombinant DNA that is incorporated into a vector, into an autonomously replicating plasmid or virus, or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or  
30 restriction endonuclease treatment) independent of other sequences. It also includes a recombinant DNA that is part of a hybrid gene encoding an additional polypeptide that is substantially free of cellular material, viral material, or culture medium (when produced by recombinant DNA techniques), or chemical precursors or other chemicals (when chemically synthesized). Moreover, an  
35 "isolated nucleic acid fragment" is a nucleic acid fragment that is not naturally occurring as a fragment and would not be found in the natural state.

As used herein, the terms "polynucleotide" or "nucleic acid molecule" are intended to include DNA molecules (e.g., cDNA or genomic DNA) and RNA molecules (e.g., mRNA) and analogs of the DNA or RNA generated using nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA. The nucleic acid may be synthesized using oligonucleotide analogs or derivatives (e.g., inosine or phosphorothioate nucleotides). Such oligonucleotides can be used, for example, to prepare nucleic acids that have altered base-pairing abilities or increased resistance to nucleases.

Another embodiment of the invention provides an isolated nucleic acid molecule which is antisense to a PEC60 nucleic acid molecule, e.g., the coding strand of a PEC60 nucleic acid molecule. Also included within the scope of the invention are the complement strands of the nucleic acid molecules described herein.

#### Sequencing errors

The sequence information as provided herein should not be so narrowly construed as to require inclusion of erroneously identified bases. The specific sequences disclosed herein can be readily used to isolate the complete gene from filamentous fungi, in particular *A. niger* which in turn can easily be subjected to further sequence analyses thereby identifying sequencing errors.

Unless otherwise indicated, all nucleotide sequences determined by sequencing a DNA molecule herein were determined using an automated DNA sequencer and all amino acid sequences of polypeptides encoded by DNA molecules determined herein were predicted by translation of a DNA sequence determined as above. Therefore, as is known in the art for any DNA sequence determined by this automated approach, any nucleotide sequence determined herein may contain some errors. Nucleotide sequences determined by automation are typically at least about 90% identical, more typically at least about 95% to at least about 99.9% identical to the actual nucleotide sequence of the sequenced DNA molecule. The actual sequence can be more precisely determined by other approaches including manual DNA sequencing methods well known in the art. As is also known in the art, a single insertion or deletion in a determined nucleotide sequence compared to the actual sequence will cause a frame shift in translation of the nucleotide sequence such that the predicted amino acid sequence encoded by a determined nucleotide sequence will be completely

different from the amino acid sequence actually encoded by the sequenced DNA molecule, beginning at the point of such an insertion or deletion.

The person skilled in the art is capable of identifying such erroneously identified bases and knows how to correct for such errors.

5

#### **Nucleic acid fragments, probes and primers**

A nucleic acid molecule according to the invention may comprise only a portion or a fragment of the nucleic acid sequence shown in SEQ ID NO:1 or SEQ ID NO:2, for example a fragment which can be used as a probe or primer or a fragment encoding a portion of a PEC60 protein. The nucleotide sequence determined from the cloning of the PEC60 gene and cDNA allows for the generation of probes and primers designed for use in identifying and/or cloning other PEC60 family members, as well as PEC60 homologues from other species. The probe/primer typically comprises substantially purified oligonucleotide which typically comprises a region of nucleotide sequence that hybridizes preferably under highly stringent conditions to at least about 12 or 15, preferably about 18 or 20, preferably about 22 or 25, more preferably about 30, 35, 40, 45, 50, 55, 60, 65, or 75 or more consecutive nucleotides of a nucleotide sequence shown in SEQ ID NO:1 or SEQ ID NO: 2 or of a functional equivalent thereof.

Probes based on the PEC60 nucleotide sequences can be used to detect transcripts or genomic PEC60 sequences encoding the same or homologous proteins for instance in other organisms. In preferred embodiments, the probe further comprises a label group attached thereto, e.g., the label group can be a radioisotope, a fluorescent compound, an enzyme, or an enzyme cofactor. Such probes can also be used as part of a diagnostic test kit for identifying cells which express a PEC60 protein.

#### **Identity & homology**

The terms "homology" or "percent identity" are used interchangeably herein. For the purpose of this invention, it is defined here that in order to determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid

sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity = number of identical positions/total number of positions (i.e. overlapping positions) x 100). Preferably, the two sequences are the same length.

The skilled person will be aware of the fact that several different computer programmes are available to determine the homology between two sequences. For instance, a comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch (J. Mol. Biol. (48):444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package (available at <http://www.gcg.com>), using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. The skilled person will appreciate that all these different parameters will yield slightly different results but that the overall percentage identity of two sequences is not significantly altered when using different algorithms.

In yet another embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available at <http://www.gcg.com>), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. In another embodiment, the percent identity two amino acid or nucleotide sequence is determined using the algorithm of E. Meyers and W. Miller (CABIOS, 4:11-17 (1989) which has been incorporated into the ALIGN program (version 2.0) (available at: <http://vega.igh.cnrs.fr/bin/align-guess.cgi>) using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

The nucleic acid and protein sequences of the present invention can further be used as a "query sequence" to perform a search against public databases to, for example, identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. (1990) J. Mol. Biol. 215:403—10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength =

12 to obtain nucleotide sequences homologous to PEC60 nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to PEC60 protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) Nucleic Acids Res. 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See <http://www.ncbi.nlm.nih.gov>.

## 10 Hybridisation

As used herein, the term "hybridizing" is intended to describe conditions for hybridization and washing under which nucleotide sequences at least about 50%, at least about 60%, at least about 70%, more preferably at least about 80%, even more preferably at least about 85% to 90%, more preferably at least 95% homologous to each other typically remain hybridized to each other.

A preferred, non-limiting example of such hybridization conditions are hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45 °C, followed by one or more washes in 1 X SSC, 0.1 % SDS at 50 °C, preferably at 55 °C, preferably at 60 °C and even more preferably at 65 °C.

Highly stringent conditions include, for example, hybridizing at 68 °C in 5x SSC/5x Denhardt's solution / 1.0% SDS and washing in 0.2x SSC/0.1% SDS at room temperature. Alternatively, washing may be performed at 42 °C.

The skilled artisan will know which conditions to apply for stringent and highly stringent hybridisation conditions. Additional guidance regarding such conditions is readily available in the art, for example, in Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, N.Y.; and Ausubel et al. (eds.), 1995, Current Protocols in Molecular Biology, (John Wiley & Sons, N.Y.).

Of course, a polynucleotide which hybridizes only to a poly A sequence (such as the 3' terminal poly(A) tract of mRNAs), or to a complementary stretch of T (or U) residues, would not be included in a polynucleotide of the invention used to specifically hybridize to a portion of a nucleic acid of the invention, since such a polynucleotide would hybridize to any nucleic acid molecule containing a poly (A) stretch or the complement thereof (e.g., practically any double-standed cDNA clone).

### **Obtaining full length DNA from other organisms**

5 In a typical approach, cDNA libraries constructed from other organisms, e.g. filamentous fungi, in particular from the species *Aspergillus* can be screened.

For example, *Aspergillus* strains can be screened for homologous PEC60 polynucleotides by Northern blot analysis. Upon detection of transcripts homologous to polynucleotides according to the invention, cDNA  
10 libraries can be constructed from RNA isolated from the appropriate strain, utilizing standard techniques well known to those of skill in the art. Alternatively, a total genomic DNA library can be screened using a probe hybridisable to a PEC60 polynucleotide according to the invention.

Homologous gene sequences can be isolated, for example, by  
15 performing PCR using two degenerate oligonucleotide primer pools designed on the basis of nucleotide sequences as taught herein.

The template for the reaction can be cDNA obtained by reverse transcription of mRNA prepared from strains known or suspected to express a polynucleotide according to the invention. The PCR product can be subcloned and  
20 sequenced to ensure that the amplified sequences represent the sequences of a new PEC60 nucleic acid sequence, or a functional equivalent thereof.

The PCR fragment can then be used to isolate a full length cDNA clone by a variety of known methods. For example, the amplified fragment can be labeled and used to screen a bacteriophage or cosmid cDNA library.  
25 Alternatively, the labeled fragment can be used to screen a genomic library.

PCR technology also can be used to isolate full length cDNA sequences from other organisms. For example, RNA can be isolated, following standard procedures, from an appropriate cellular or tissue source. A reverse transcription reaction can be performed on the RNA using an oligonucleotide  
30 primer specific for the most 5' end of the amplified fragment for the priming of first strand synthesis.

The resulting RNA/DNA hybrid can then be "tailed" (e.g., with guanines) using a standard terminal transferase reaction, the hybrid can be digested with RNase H, and second strand synthesis can then be primed (e.g.,  
35 with a poly-C primer). Thus, cDNA sequences upstream of the amplified fragment can easily be isolated. For a review of useful cloning strategies, see e.g., Sambrook et al., supra; and Ausubel et al., supra.

## Vectors

Another aspect of the invention pertains to vectors, preferably  
5 expression vectors, containing a nucleic acid encoding a PEC60 protein or a  
functional equivalent thereof. As used herein, the term "vector" refers to a nucleic  
acid molecule capable of transporting another nucleic acid to which it has been  
linked. One type of vector is a "plasmid", which refers to a circular double stranded  
10 DNA loop into which additional DNA segments can be ligated. Another type of  
vector is a viral vector, wherein additional DNA segments can be ligated into the  
viral genome. Certain vectors are capable of autonomous replication in a host cell  
into which they are introduced (e.g., bacterial vectors having a bacterial origin of  
replication and episomal mammalian vectors). Other vectors (e.g., non-episomal  
15 mammalian vectors) are integrated into the genome of a host cell upon  
introduction into the host cell, and thereby are replicated along with the host  
genome. Moreover, certain vectors are capable of directing the expression of  
genes to which they are operatively linked. Such vectors are referred to herein as  
"expression vectors". In general, expression vectors of utility in recombinant DNA  
20 techniques are often in the form of plasmids. The terms "plasmid" and "vector" can  
be used interchangeably herein as the plasmid is the most commonly used form  
of vector. However, the invention is intended to include such other forms of  
expression vectors, such as viral vectors (e.g., replication defective retroviruses,  
adenoviruses and adeno-associated viruses), which serve equivalent functions.

The recombinant expression vectors of the invention comprise a  
25 nucleic acid of the invention in a form suitable for expression of the nucleic acid in  
a host cell, which means that the recombinant expression vector includes one or  
more regulatory sequences, selected on the basis of the host cells to be used for  
expression, which is operatively linked to the nucleic acid sequence to be  
expressed. Within a recombinant expression vector, "operatively linked" is  
30 intended to mean that the nucleotide sequence of interest is linked to the  
regulatory sequence(s) in a manner which allows for expression of the nucleotide  
sequence (e.g., in an *in vitro* transcription/translation system or in a host cell when  
the vector is introduced into the host cell). The term "regulatory sequence" is  
intended to include promoters, enhancers and other expression control elements  
35 (e.g., polyadenylation signal). Such regulatory sequences are described, for  
example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185,  
Academic Press, San Diego, CA (1990). Regulatory sequences include those



which direct constitutive expression of a nucleotide sequence in many types of host cells and those which direct expression of the nucleotide sequence only in a certain host cell (e.g. tissue-specific regulatory sequences). It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, encoded by nucleic acids as described herein (e.g. PEC60 proteins, mutant forms of PEC60 proteins, fragments, variants or functional equivalents thereof, fusion proteins, etc.).

The recombinant expression vectors of the invention can be designed for expression of PEC60 proteins in prokaryotic or eukaryotic cells. For example, PEC60 proteins can be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus expression vectors) yeast cells or mammalian cells. Suitable host cells are discussed further in Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Alternatively, the recombinant expression vector can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

Expression vectors useful in the present invention include chromosomal-, episomal- and virus-derived vectors e.g., vectors derived from bacterial plasmids, bacteriophage, yeast episome, yeast chromosomal elements, viruses such as baculoviruses, papova viruses, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids.

The DNA insert should be operatively linked to an appropriate promoter, such as the phage lambda PL promoter, the *E. coli* lac, trp and tac promoters, the SV40 early and late promoters and promoters of retroviral LTRs, to name a few. Other suitable promoters will be known to the skilled person. In a specific embodiment, promoters are preferred that are capable of directing a high expression level of pectinases in filamentous fungi. Such promoters are known in the art. The expression constructs may contain sites for transcription initiation, termination, and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs will include a translation initiating AUG at the beginning and a termination codon

appropriately positioned at the end of the polypeptide to be translated.

Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (e.g., DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, transduction, infection, lipofection, cationic lipid-mediated transfection or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook, et al. (*Molecular Cloning: A Laboratory Manual*, 2<sup>nd</sup>, ed. Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), Davis et al., *Basic Methods in Molecular Biology* (1986) and other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (e.g., resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker can be introduced into a host cell on the same vector as that encoding a PEC60 protein or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (e.g. cells that have incorporated the selectable marker gene will survive, while the other cells die).

Expression of proteins in prokaryotes is often carried out in *E. coli* with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, e.g. to the amino terminus of the recombinant protein. Such fusion vectors typically serve three purposes: 1) to increase expression of recombinant protein; 2) to increase the solubility of the recombinant protein; and 3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and

enterokinase.

As indicated, the expression vectors will preferably contain selectable markers. Such markers include dihydrofolate reductase or neomycin resistance for eukarotic cell culture and tetracycline or ampicilling resistance for culturing in *E. coli* and other bacteria. Representative examples of appropriate host include bacterial cells, such as *E. coli*, *Streptomyces* and *Salmonella typhimurium*; fungal cells, such as yeast; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9; animal cells such as CHO, COS and Bowes melanoma; and plant cells. Appropriate culture mediums and conditions for the above-described host cells are known in the art.

Among vectors preferred for use in bacteria are pQE70, pQE60 and PQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16A, pNH18A, pNH46A, available from Stratagene; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia. Among preferred eukaryotic vectors are PWLNEO, pSV2CAT, pOG44, pZT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. Other suitable vectors will be readily apparent to the skilled artisan.

Among known bacterial promoters for use in the present invention include *E. coli* lacI and lacZ promoters, the T3 and T7 promoters, the gpt promoter, the lambda PR, PL promoters and the trp promoter, the HSV thymidine kinase promoter, the early and late SV40 promoters, the promoters of retroviral LTRs, such as those of the Rous sarcoma virus ("RSV"), and metallothionein promoters, such as the mouse metallothionein-I promoter.

Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act to increase transcriptional activity of a promoter in a given host cell-type. Examples of enhancers include the SV40 enhancer, which is located on the late side of the replication origin at bp 100 to 270, the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

For secretion of the translated protein into the lumen of the endoplasmic reticulum, into the periplasmic space or into the extracellular environment, appropriate secretion signal may be incorporated into the expressed polypeptide. The signals may be endogenous to the polypeptide or they may be heterologous signals.

The polypeptide may be expressed in a modified form, such as a fusion protein, and may include not only secretion signals but also additional heterologous functional regions. Thus, for instance, a region of additional amino acids, particularly charged amino acids, may be added to the N-terminus of the polypeptide to improve stability and persistence in the host cell, during purification or during subsequent handling and storage. Also, peptide moieties may be added to the polypeptide to facilitate purification.

### **Polypeptides according to the invention**

The invention provides an isolated polypeptide having the amino acid sequence according to SEQ ID NO: 3, an amino acid sequence obtainable by expressing the polynucleotide of SEQ ID NO: 1 in an appropriate host, as well as an amino acid sequence obtainable by expressing the polynucleotide sequences of SEQ ID NO: 2 in an appropriate host. Also, a peptide or polypeptide comprising a functional equivalent of the above polypeptides is comprised within the present invention. The above polypeptides are collectively comprised in the term "polypeptides according to the invention"

The terms "peptide" and "oligopeptide" are considered synonymous (as is commonly recognized) and each term can be used interchangeably as the context requires to indicate a chain of at least two amino acids coupled by peptidyl linkages. The word "polypeptide" is used herein for chains containing more than seven amino acid residues. All oligopeptide and polypeptide formulas or sequences herein are written from left to right and in the direction from amino terminus to carboxy terminus. The one-letter code of amino acids used herein is commonly known in the art and can be found in Sambrook, et al. (*Molecular Cloning: A Laboratory Manual, 2<sup>nd</sup>, ed. Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989*)

By "isolated" polypeptide or protein is intended a polypeptide or protein removed from its native environment. For example, recombinantly produced polypeptides and proteins expressed in host cells are considered isolated for the purpose of the invention as are native or recombinant polypeptides which have been substantially purified by any suitable technique such as, for example, the single-step purification method disclosed in Smith and Johnson, Gene 67:31-40 (1988).

The PEC60 pectinase according to the invention can be recovered and purified from recombinant cell cultures by well-known methods

including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography ("HPLC") is employed for purification.

Polypeptides of the present invention include naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect and mammalian cells. Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. In addition, polypeptides of the invention may also include an initial modified methionine residue, in some cases as a result of host-mediated processes.

#### **Protein fragments**

The invention also features biologically active fragments of the polypeptides according to the invention.

Biologically active fragments of a polypeptide of the invention include polypeptides comprising amino acid sequences sufficiently identical to or derived from the amino acid sequence of the PEC60 protein (e.g., the amino acid sequence of SEQ ID NO: 3), which include fewer amino acids than the full length protein, and exhibit at least one biological activity of the corresponding full-length protein. Typically, biologically active fragments comprise a domain or motif with at least one activity of the PEC60 protein. A biologically active fragment of a protein of the invention can be a polypeptide which is, for example, 10, 25, 50, 100 or more amino acids in length. Moreover, other biologically active portions, in which other regions of the protein are deleted, can be prepared by recombinant techniques and evaluated for one or more of the biological activities of the native form of a polypeptide of the invention.

The invention also features nucleic acid fragments which encode the above biologically active fragments of the PEC60 protein.

#### **Fusion proteins**

The proteins of the present invention or functional equivalents thereof, e.g., biologically active portions thereof, can be operatively linked to a

non-PEC60 polypeptide (e.g., heterologous amino acid sequences) to form fusion proteins. As used herein, a PEC60 "chimeric protein" or "fusion protein" comprises a PEC60 polypeptide operatively linked to a non-PEC60 polypeptide. A "PEC60 polypeptide" refers to a polypeptide having an amino acid sequence  
5 corresponding to PEC60, whereas a "non-PEC60 polypeptide" refers to a polypeptide having an amino acid sequence corresponding to a protein which is not substantially homologous to the PEC60 protein, e.g., a protein which is different from the PEC60 protein and which is derived from the same or a different organism. Within a PEC60 fusion protein the PEC60 polypeptide can correspond  
10 to all or a portion of a PEC60 protein. In a preferred embodiment, a PEC60 fusion protein comprises at least one biologically active fragment of a PEC60 protein. In another preferred embodiment, a PEC60 fusion protein comprises at least two biologically active portions of a PEC60 protein. Within the fusion protein, the term "operatively linked" is intended to indicate that the PEC60 polypeptide and the  
15 non-PEC60 polypeptide are fused in-frame to each other. The non-PEC60 polypeptide can be fused to the N-terminus or C-terminus of the PEC60 polypeptide.

For example, in one embodiment, the fusion protein is a GST-PEC60 fusion protein in which the PEC60 sequences are fused to the C-terminus  
20 of the GST sequences. Such fusion proteins can facilitate the purification of recombinant PEC60. In another embodiment, the fusion protein is a PEC60 protein containing a heterologous signal sequence at its N-terminus. In certain host cells (e.g., mammalian and Yeast host cells), expression and/or secretion of PEC60 can be increased through use of a heterologous signal sequence.

25 In another example, the gp67 secretory sequence of the baculovirus envelope protein can be used as a heterologous signal sequence (*Current Protocols in Molecular Biology*, Ausubel et al., eds., John Wiley & Sons, 1992). Other examples of eukaryotic heterologous signal sequences include the secretory sequences of melittin and human placental alkaline phosphatase  
30 (Stratagene; La Jolla, California). In yet another example, useful prokaryotic heterologous signal sequences include the phoA secretory signal (Sambrook et al., *supra*) and the protein A secretory signal (Pharmacia Biotech; Piscataway, New Jersey).

A signal sequence can be used to facilitate secretion and  
35 isolation of a protein or polypeptide of the invention. Signal sequences are typically characterized by a core of hydrophobic amino acids which are generally

cleaved from the mature protein during secretion in one or more cleavage events. Such signal peptides contain processing sites that allow cleavage of the signal sequence from the mature proteins as they pass through the secretory pathway.

The signal sequence directs secretion of the protein, such as from a eukaryotic

5 host into which the expression vector is transformed, and the signal sequence is subsequently or concurrently cleaved. The protein can then be readily purified from the extracellular medium by art recognized methods. Alternatively, the signal sequence can be linked to the protein of interest using a sequence which

facilitates purification, such as with a GST domain. Thus, for instance, the

10 sequence encoding the polypeptide may be fused to a marker sequence, such as a sequence encoding a peptide, which facilitates purification of the fused polypeptide. In certain preferred embodiments of this aspect of the invention, the

marker sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (Qiagen, Inc.), among others, many of which are commercially available.

15 As described in *Gentz et al, Proc. Natl. Acad. Sci. USA 86:821-824 (1989)*, for instance, hexa-histidine provides for convenient purification of the fusion protein.

The HA tag is another peptide useful for purification which corresponds to an epitope derived of influenza hemagglutinin protein, which has been described by Wilson *et al.*, *Cell 37:767 (1984)*, for instance.

20 Preferably, a PEC60 chimeric or fusion protein of the invention

is produced by standard recombinant DNA techniques. For example, DNA fragments coding for the different polypeptide sequences are ligated together in-frame in accordance with conventional techniques, for example by employing

blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to

25 provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In

another embodiment, the fusion gene can be synthesized by conventional

techniques including automated DNA synthesizers. Alternatively, PCR

amplification of gene fragments can be carried out using anchor primers which

30 give rise to complementary overhangs between two consecutive gene fragments

which can subsequently be annealed and reamplified to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel

*et al.* John Wiley & Sons: 1992). Moreover, many expression vectors are commercially available that already encode a fusion moiety (e.g, a GST

polypeptide). A PEC60-encoding nucleic acid can be cloned into such an

35 expression vector such that the fusion moiety is linked in-frame to the PEC60

protein.

### **Functional equivalents**

5                   The terms "functional equivalents" and "functional variants" are used interchangeably herein. Functional equivalents of PEC60 DNA are isolated DNA fragments that encode a polypeptide that exhibits a particular function of the PEC60 *A. niger* pectinase as defined herein. A functional equivalent of a PEC60 polypeptide according to the invention is a polypeptide that exhibits at least one  
10                   function of an *A. niger* pectinase as defined herein. Functional equivalents therefore also encompass biologically active fragments.

                  Functional protein or polypeptide equivalents may contain only conservative substitutions of one or more amino acids of SEQ ID NO: 3 or substitutions, insertions or deletions of non-essential amino acids. Accordingly, a  
15                   non-essential amino acid is a residue that can be altered in SEQ ID NO: 3 without substantially altering the biological function. For example, amino acid residues that are conserved among the PEC60 proteins of the present invention, are predicted to be particularly unamenable to alteration. Furthermore, amino acids conserved among the PEC60 proteins according to the present invention and  
20                   other pectinases are not likely to be amenable to alteration.

                  The term "conservative substitution" is intended to mean that a substitution in which the amino acid residue is replaced with an amino acid residue having a similar side chain. These families are known in the art and include amino acids with basic side chains (e.g. lysine, arginine and histidine),  
25                   acidic side chains (e.g. aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagines, glutamine, serine, threonine, tyrosine, cysteine), non-polar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine tryptophan,  
30                   histidine).

                  Functional nucleic acid equivalents may typically contain silent mutations or mutations that do not alter the biological function of encoded polypeptide. Accordingly, the invention provides nucleic acid molecules encoding PEC60 proteins that contain changes in amino acid residues that are not essential  
35                   for a particular biological activity. Such PEC60 proteins differ in amino acid sequence from SEQ ID NO: 3 yet retain at least one biological activity. In one embodiment the isolated nucleic acid molecule comprises a nucleotide sequence



encoding a protein, wherein the protein comprises a substantially homologous amino acid sequence of at least about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more homologous to the amino acid sequence shown in SEQ ID NO: 3.

5                   For example, guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie, J.U. et al., Science 247:1306-1310 (1990) wherein the authors indicate that there are two main approaches for studying the tolerance of an amino acid sequence to change. The first method relies on the process of evolution, in which mutations are either accepted or  
10                   rejected by natural selection. The second approach uses genetic engineering to introduce amino acid changes at specific positions of a cloned gene and selects or screens to identify sequences that maintain functionality. As the authors state, these studies have revealed that proteins are surprisingly tolerant of amino acid substitutions. The authors further indicate which changes are likely to be  
15                   permissive at a certain position of the protein. For example, most buried amino acid residues require non-polar side chains, whereas few features of surface side chains are generally conserved. Other such phenotypically silent substitutions are described in Bowie et al, supra, and the references cited therein.

                  An isolated nucleic acid molecule encoding a PEC60 protein  
20                   homologous to the protein according to SEQ ID NO: 3 can be created by introducing one or more nucleotide substitutions, additions or deletions into the coding nucleotide sequences according to SEQ ID NO: 1 or SEQ ID NO: 2 such that one or more amino acid substitutions, deletions or insertions are introduced into the encoded protein. Such mutations may be introduced by standard  
25                   techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis.

                  The term "functional equivalents" also encompasses orthologues of the A. niger PEC60 protein. Orthologues of the A. niger PEC60 protein are proteins that can be isolated from other strains or species and possess a similar or identical biological activity. Such orthologues can readily be identified  
30                   as comprising an amino acid sequence that is substantially homologous to SEQ ID NO: 3.

                  As defined herein, the term "substantially homologous" refers to a first amino acid or nucleotide sequence which contains a sufficient or minimum number of identical or equivalent (e.g., with similar side chain) amino acids or  
35                   nucleotides to a second amino acid or nucleotide sequence such that the first and the second amino acid or nucleotide sequences have a common domain. For

example, amino acid or nucleotide sequences which contain a common domain having about 60%, preferably 65%, more preferably 70%, even more preferably 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identity or more are defined herein as sufficiently identical.

5                   Also, nucleic acids encoding other PEC60 family members, which thus have a nucleotide sequence that differs from SEQ ID NO: 1 or SEQ ID NO: 2, are within the scope of the invention. Moreover, nucleic acids encoding PEC60 proteins from different species which thus have a nucleotide sequence which differs from SEQ ID NO: 1 or SEQ ID NO: 2 are within the scope of the  
10                  invention.

                  Nucleic acid molecules corresponding to variants (e.g. natural allelic variants) and homologues of the PEC60 DNA of the invention can be isolated based on their homology to the PEC60 nucleic acids disclosed herein using the cDNAs disclosed herein or a suitable fragment thereof, as a  
15                  hybridisation probe according to standard hybridisation techniques preferably under highly stringent hybridisation conditions.

                  In addition to naturally occurring allelic variants of the PEC60 sequence, the skilled person will recognise that changes can be introduced by mutation into the nucleotide sequences of SEQ ID NO: 1 or SEQ ID NO: 2 thereby  
20                  leading to changes in the amino acid sequence of the PEC60 protein without substantially altering the function of the PEC60 protein.

                  In another aspect of the invention, improved PEC60 proteins are provided. Improved PEC60 proteins are proteins wherein at least one biological activity is improved. Such proteins may be obtained by randomly introducing  
25                  mutations along all or part of the PEC60 coding sequence, such as by saturation mutagenesis, and the resulting mutants can be expressed recombinantly and screened for biological activity. For instance, the art provides for standard assays for measuring the enzymatic activity of pectinases and thus improved proteins may easily be selected.

30                  In a preferred embodiment the PEC60 protein has an amino acid sequence according to SEQ ID NO: 3. In another embodiment, the PEC60 polypeptide is substantially homologous to the amino acid sequence according to SEQ ID NO: 3 and retains at least one biological activity of a polypeptide according to SEQ ID NO: 3, yet differs in amino acid sequence due to natural  
35                  variation or mutagenesis as described above.

                  In a further preferred embodiment, the PEC60 protein has an

amino acid sequence encoded by an isolated nucleic acid fragment capable of hybridising to a nucleic acid according to SEQ ID NO: 1 or SEQ ID NO: 2, preferably under highly stringent hybridisation conditions.

Accordingly, the PEC60 protein is a protein which comprises an amino acid sequence at least about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or more homologous to the amino acid sequence shown in SEQ ID NO: 3 and retains at least one functional activity of the polypeptide according to SEQ ID NO: 3.

Functional equivalents of a protein according to the invention can also be identified e.g. by screening combinatorial libraries of mutants, e.g. truncation mutants, of the protein of the invention for pectinase activity. In one embodiment, a variegated library of variants is generated by combinatorial mutagenesis at the nucleic acid level. A variegated library of variants can be produced by, for example, enzymatically ligating a mixture of synthetic oligonucleotides into gene sequences such that a degenerate set of potential protein sequences is expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g., for phage display). There are a variety of methods that can be used to produce libraries of potential variants of the polypeptides of the invention from a degenerate oligonucleotide sequence. Methods for synthesizing degenerate oligonucleotides are known in the art (see, e.g., Narang (1983) Tetrahedron 39:3; Itakura et al. (1984) Annu. Rev. Biochem. 53:323; Itakura et al. (1984) Science 198:1056; Ike et al. (1983) Nucleic Acid Res. 11:477).

In addition, libraries of fragments of the coding sequence of a polypeptide of the invention can be used to generate a variegated population of polypeptides for screening a subsequent selection of variants. For example, a library of coding sequence fragments can be generated by treating a double stranded PCR fragment of the coding sequence of interest with a nuclease under conditions wherein nicking occurs only about once per molecule, denaturing the double stranded DNA, renaturing the DNA to form double stranded DNA which can include sense/antisense pairs from different nicked products, removing single stranded portions from reformed duplexes by treatment with S1 nuclease, and ligating the resulting fragment library into an expression vector. By this method, an expression library can be derived which encodes N-terminal and internal fragments of various sizes of the protein of interest.

Several techniques are known in the art for screening gene

products of combinatorial libraries made by point mutations of truncation, and for screening cDNA libraries for gene products having a selected property. The most widely used techniques, which are amenable to high through-put analysis, for screening large gene libraries typically include cloning the gene library into  
5 replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates isolation of the vector encoding the gene whose product was detected. Recursive ensemble mutagenesis (REM), a technique which enhances the frequency of functional mutants in the libraries, can  
10 be used in combination with the screening assays to identify variants of a protein of the invention (Arkin and Yourvan (1992) Proc. Natl. Acad. Sci. USA 89:7811-7815; Delgrave et al. (1993) Protein Engineering 6(3):327-331).

In addition to the PEC60 gene sequence shown in SEQ ID NO: 1, it will be apparent for the person skilled in the art that DNA sequence  
15 polymorphisms that may lead to changes in the amino acid sequence of the PEC60 protein may exist within a given population. Such genetic polymorphisms may exist in cells from different populations or within a population due to natural allelic variation. Allelic variants may also include functional equivalents.

Fragments of a polynucleotide according to the invention may  
20 also comprise polynucleotides not encoding functional polypeptides. Such polynucleotides may function as probes or primers for a PCR reaction.

Nucleic acids according to the invention irrespective of whether they encode functional or non-functional polypeptides, can be used as hybridization probes or polymerase chain reaction (PCR) primers. Uses of the  
25 nucleic acid molecules of the present invention that do not encode a polypeptide having a PEC60 activity include, inter alia, (1) isolating the gene encoding the PEC60 protein, or allelic variants thereof from a cDNA library e.g. from other organisms than *A. niger*; (2) in situ hybridization (e.g. FISH) to metaphase chromosomal spreads to provide precise chromosomal location of the PEC60  
30 gene as described in Verma et al., Human Chromosomes: a Manual of Basic Techniques, Pergamon Press, New York (1988); (3) Northern blot analysis for detecting expression of PEC60 mRNA in specific tissues and/or cells and 4) probes and primers that can be used as a diagnostic tool to analyse the presence of a nucleic acid hybridisable to the PEC60 probe in a given biological (e.g. tissue)  
35 sample.

Also encompassed by the invention is a method of obtaining a

functional equivalent of a PEC60 gene or cDNA. Such a method entails obtaining a labelled probe that includes an isolated nucleic acid which encodes all or a portion of the sequence according to SEQ ID NO: 3 or a variant thereof; screening a nucleic acid fragment library with the labelled probe under conditions that allow  
5 hybridisation of the probe to nucleic acid fragments in the library, thereby forming nucleic acid duplexes, and preparing a full-length gene sequence from the nucleic acid fragments in any labelled duplex to obtain a gene related to the PEC60 gene.

In one embodiment, a PEC60 nucleic acid of the invention is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%,  
10 97%, 98%, 99%, or more homologous to a nucleic acid sequence shown in SEQ ID NO: 1, SEQ ID NO: 2 or the complement thereof.

In another preferred embodiment a PEC60 polypeptide of the invention is at least 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or more homologous to the amino acid  
15 sequence shown in SEQ ID NO: 3.

### **Host cells**

In another embodiment, the invention features cells, e.g.,  
20 transformed host cells or recombinant host cells that contain a nucleic acid encompassed by the invention. A "transformed cell" or "recombinant cell" is a cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a nucleic acid according to the invention. Both prokaryotic and eukaryotic cells are included, e.g., bacteria, fungi, yeast, and the  
25 like, especially preferred are cells from filamentous fungi, in particular *Aspergillus niger*.

A host cell can be chosen that modulates the expression of the inserted sequences, or modifies and processes the gene product in a specific, desired fashion. Such modifications (e.g., glycosylation) and processing (e.g.,  
30 cleavage) of protein products may facilitate optimal functioning of the protein.

Various host cells have characteristic and specific mechanisms for post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems familiar to those of skill in the art of molecular biology and/or microbiology can be chosen to ensure the desired and  
35 correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells that possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product can

be used. Such host cells are well known in the art.

Host cells also include, but are not limited to, mammalian cell lines such as CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, and choroid plexus cell lines.

5                   If desired, the polypeptides according to the invention can be produced by a stably-transfected cell line. A number of vectors suitable for stable transfection of mammalian cells are available to the public, methods for constructing such cell lines are also publicly known, e.g., in Ausubel et al. (*supra*).

## 10    **Antibodies**

The invention further features antibodies, such as monoclonal or polyclonal antibodies, that specifically bind PEC60 proteins according to the invention.

15                   As used herein, the term "antibody" (Ab) or "monoclonal antibody" (Mab) is meant to include intact molecules as well as antibody fragments (such as, for example, Fab and F(ab')<sub>2</sub> fragments) which are capable of specifically binding to PEC60 protein. Fab and F(ab')<sub>2</sub> fragments lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have  
20   less non-specific tissue binding of an intact antibody (Wahl *et al.*, *J. Nucl. Med.* 24:316-325 (1983)). Thus, these fragments are preferred.

The antibodies of the present invention may be prepared by any of a variety of methods. For example, cells expressing the PEC60 protein or an antigenic fragment thereof can be administered to an animal in order to induce the  
25   production of sera containing polyclonal antibodies. In a preferred method, a preparation of PEC60 protein is prepared and purified to render it substantially free of natural contaminants. Such a preparation is then introduced into an animal in order to produce polyclonal antisera of greater specific activity.

In the most preferred method, the antibodies of the present  
30   invention are monoclonal antibodies (or PEC60 protein binding fragments thereof). Such monoclonal antibodies can be prepared using hybridoma technology (Kohler *et al.*, *Nature* 256:495 (1975); Kohler *et al.*, *Eur. J. Immunol.* 6:511 (1976); Hammerling *et al.*, In: *Monoclonal Antibodies and T-Cell Hybridomas*, Elsevier, N.Y., pp. 563-681 (1981)). In general, such procedures  
35   involve immunizing an animal (preferably a mouse) with a PEC60 protein antigen or, with a PEC60 protein expressing cell. The splenocytes of such mice are extracted and fused with a suitable myeloma cell line. Any suitable myeloma cell

line may be employed in accordance with the present invention; however, it is preferably to employ the parent myeloma cell line (SP<sub>2</sub>O), available from the American Type Culture Collection, Rockville, Maryland. After fusion, the resulting hybridoma cells are selectively maintained in HAT medium, and then cloned by  
5 limiting dilution as described by Wands *et al.* (*Gastro-enterology* 80:225-232 (1981)). The hybridoma cells obtained through such a selection are then assayed to identify clones which secrete antibodies capable of binding the PEC60 protein antigen. In general, the polypeptides can be coupled to a carrier protein, such as KLH, as described in Ausubel *et al.*, supra, mixed with an adjuvant, and injected  
10 into a host mammal.

In particular, various host animals can be immunized by injection of a polypeptide of interest. Examples of suitable host animals include rabbits, mice, guinea pigs, and rats. Various adjuvants can be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), adjuvant mineral gels such as aluminum  
15 hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*. Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of the immunized animals.  
20

Such antibodies can be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD, and any subclass thereof. The hybridomas producing the mAbs of this invention can be cultivated *in vitro* or *in vivo*.

Once produced, polyclonal or monoclonal antibodies are tested  
25 for specific recognition of a PEC60 polypeptide or functional equivalent thereof in an immunoassay, such as a Western blot or immunoprecipitation analysis using standard techniques, e.g., as described in Ausubel *et al.*, supra. Antibodies that specifically bind to PEC60 proteins or functional equivalents thereof are useful in the invention. For example, such antibodies can be used in an immunoassay to  
30 detect PEC60 in pathogenic or non-pathogenic strains of *Aspergillus* (e.g., in *Aspergillus* extracts).

Preferably, antibodies of the invention are produced using fragments of the PEC60 polypeptides that appear likely to be antigenic, by criteria such as high frequency of charged residues. For example, such fragments may be  
35 generated by standard techniques of PCR, and then cloned into the pGEX expression vector (Ausubel *et al.*, supra). Fusion proteins may then be expressed

in *E. coli* and purified using a glutathione agarose affinity matrix as described in Ausubel, et al., supra. If desired, several (e.g., two or three) fusions can be generated for each protein, and each fusion can be injected into at least two rabbits. Antisera can be raised by injections in a series, typically including at least  
5 three booster injections. Typically, the antisera are checked for their ability to immunoprecipitate a recombinant PEC60 polypeptide or functional equivalents thereof whereas unrelated proteins may serve as a control for the specificity of the immune reaction.

Alternatively, techniques described for the production of single  
10 chain antibodies (U.S. Patent 4,946,778 and 4,704,692) can be adapted to produce single chain antibodies against a PEC60 polypeptide or functional equivalents thereof. Kits for generating and screening phage display libraries are commercially available e.g. from Pharmacia.

Additionally, examples of methods and reagents particularly  
15 amenable for use in generating and screening antibody display library can be found in, for example, U.S. Patent No. 5,223, 409; PCT Publication No. WO 92/18619; PCT Publication No. WO 91/17271; PCT Publication No. WO 20791; PCT Publication No. WO 92/20791; PCT Publication No. WO 92/15679; PCT Publication No. WO 93/01288; PCT Publication No. WO  
20 92/01047; PCT Publication No. WO 92/09690; PCT Publication No. WO 90/02809; Fuchs et al. (1991) *Bio/Technology* 9:1370-1372; Hay et al. (1992) *Hum. Antibod. Hybridomas* 3:81-85; Huse et al. (1989) *Science* 246:1275-1281; Griffiths et al. (1993) *EMBO J.* 12:725-734.

Polyclonal and monoclonal antibodies that specifically bind  
25 PEC60 polypeptides or functional equivalents thereof can be used, for example, to detect expression of a PEC60 gene or a functional equivalent thereof e.g. in another strain of *Aspergillus*. For example, PEC60 polypeptide can be readily detected in conventional immunoassays of *Aspergillus* cells or extracts. Examples of suitable assays include, without limitation, Western blotting, ELISAs,  
30 radioimmune assays, and the like.

By "specifically binds" is meant that an antibody recognizes and binds a particular antigen, e.g., a PEC60 polypeptide, but does not substantially recognize and bind other unrelated molecules in a sample.

Antibodies can be purified, for example, by affinity  
35 chromatography methods in which the polypeptide antigen is immobilized on a resin.



An antibody directed against a polypeptide of the invention (e.g., monoclonal antibody) can be used to isolate the polypeptide by standard techniques, such as affinity chromatography or immunoprecipitation. Moreover, such an antibody can be used to detect the protein (e.g., in a cellular lysate or cell supernatant) in order to evaluate the abundance and pattern of expression of the polypeptide. The antibodies can also be used diagnostically to monitor protein levels in cells or tissue as part of a clinical testing procedure, e.g., to, for example, determine the efficacy of a given treatment regimen or in the diagnosis of Aspergillosis..

Detection can be facilitated by coupling the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase,  $\beta$ -galactosidase, or acetylcholinesterase; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive materials include  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{35}\text{S}$  or  $^3\text{H}$ .

Preferred epitopes encompassed by the antigenic peptide are regions that are located on the surface of the protein, e.g., hydrophilic regions. Hydrophobicity plots of the proteins of the invention can be used to identify hydrophilic regions.

The antigenic peptide of a protein of the invention comprises at least 7 (preferably 10, 15, 20, or 30) contiguous amino acid residues of the amino acid sequence of SEQ ID NO: 3 and encompasses an epitope of the protein such that an antibody raised against the peptide forms a specific immune complex with the protein.

Preferred epitopes encompassed by the antigenic peptide are regions of PEC60 that are located on the surface of the protein, e.g., hydrophilic regions, hydrophobic regions, alpha regions, beta regions, coil regions, turn regions and flexible regions.

### **Immunoassays**

Qualitative or quantitative determination of a polypeptide

according to the present invention in a biological sample can occur using any art-known method. Antibody-based techniques provide special advantages for assaying specific polypeptide levels in a biological sample.

5 In these, the specific recognition is provided by the primary antibody (polyclonal or monoclonal) but the secondary detection system can utilize fluorescent, enzyme, or other conjugated secondary antibodies. As a result, an immunocomplex is obtained.

Accordingly, the invention provides a method for diagnosing whether a certain organism is infected with *Aspergillus* comprising the steps of:

- 10
- Isolating a biological sample from said organism suspected to be infected with *Aspergillus*,
  - reacting said biological sample with an antibody according to the invention,
  - determining whether immune complexes are formed.

15 Tissues can also be extracted, e.g., with urea and neutral detergent, for the liberation of protein for Western-blot or dot/slot assay. This technique can also be applied to body fluids.

Other antibody-based methods useful for detecting PEC60 gene expression include immunoassays, such as the enzyme linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA). For example, PEC60-specific  
20 monoclonal antibodies can be used both as an immunoabsorbent and as an enzyme-labeled probe to detect and quantify the PEC60 protein. The amount of PEC60 protein present in the sample can be calculated by reference to the amount present in a standard preparation using a linear regression computer algorithm. In another ELISA assay, two distinct specific monoclonal antibodies can  
25 be used to detect PEC60 protein in a biological fluid. In this assay, one of the antibodies is used as the immuno-absorbent and the other as the enzyme-labeled probe.

The above techniques may be conducted essentially as a "one-step" or "two-step" assay. The "one-step" assay involves contacting PEC60  
30 protein with immobilized antibody and, without washing, contacting the mixture with the labeled antibody. The "two-step" assay involves washing before contacting the mixture with the labeled antibody. Other conventional methods may also be employed as suitable. It is usually desirable to immobilize one component of the assay system on a support, thereby allowing other components of the  
35 system to be brought into contact with the component and readily removed from the sample.

Suitable enzyme labels include, for example, those from the oxidase group, which catalyze the production of hydrogen peroxide by reacting with substrate. Activity of an oxidase label may be assayed by measuring the concentration of hydrogen peroxide formed by the enzyme-labelled antibody/substrate reaction.

Besides enzymes, other suitable labels include radioisotopes, such as iodine ( $^{125}\text{I}$ ,  $^{127}\text{I}$ ), carbon ( $^{14}\text{C}$ ), sulphur ( $^{35}\text{S}$ ), tritium ( $^3\text{H}$ ), indium ( $^{112}\text{In}$ ), and technetium ( $^{99\text{m}}\text{Tc}$ ), and fluorescent labels, such as fluorescein and rhodamine, and biotin.

Specific binding of a test compound to a PEC60 polypeptide can be detected, for example, in vitro by reversibly or irreversibly immobilizing the PEC60 polypeptide on a substrate, e.g., the surface of a well of a 96-well polystyrene microtitre plate. Methods for immobilizing polypeptides and other small molecules are well known in the art. For example, the microtitre plates can be coated with a PEC60 polypeptide by adding the polypeptide in a solution (typically, at a concentration of 0.05 to 1 mg/ml in a volume of 1-100  $\mu\text{l}$ ) to each well, and incubating the plates at room temperature to 37 °C for 0.1 to 36 hours. Polypeptides that are not bound to the plate can be removed by shaking the excess solution from the plate, and then washing the plate (once or repeatedly) with water or a buffer. Typically, the polypeptide is contained in water or a buffer. The plate is then washed with a buffer that lacks the bound polypeptide. To block the free protein-binding sites on the plates, the plates are blocked with a protein that is unrelated to the bound polypeptide. For example, 300  $\mu\text{l}$  of bovine serum albumin (BSA) at a concentration of 2 mg/ml in Tris-HCl is suitable. Suitable substrates include those substrates that contain a defined cross-linking chemistry (e.g., plastic substrates, such as polystyrene, styrene, or polypropylene substrates from Corning Costar Corp. (Cambridge, MA), for example) . If desired, a beaded particle, e.g., beaded agarose or beaded sepharose, can be used as the substrate.

Binding of the test compound to the polypeptides according to the invention can be detected by any of a variety of artknown methods. For example, a specific antibody can be used in an immunoassay. If desired, the antibody can be labeled (e.g., fluorescently or with a radioisotope) and detected directly (see, e.g., West and McMahon, J. Cell Biol. 74:264, 1977). Alternatively, a second antibody can be used for detection (e.g., a labeled antibody that binds the Fc portion of an anti-AN97 antibody). In an alternative detection method, the

PEC60 polypeptide is labeled, and the label is detected (e.g., by labeling aPEC60 polypeptide with a radioisotope, fluorophore, chromophore, or the like). In still another method, the PEC60 polypeptide is produced as a fusion protein with a protein that can be detected optically, e.g., green fluorescent protein (which can  
5 be detected under UV light). In an alternative method, the PEC60 polypeptide can be covalently attached to or fused with an enzyme having a detectable enzymatic activity, such as horse radish peroxidase, alkaline phosphatase, a-galactosidase, or glucose oxidase. Genes encoding all of these enzymes have been cloned and are readily available for use by those of skill in the art. If desired, the fusion protein  
10 can include an antigen, and such an antigen can be detected and measured with a polyclonal or monoclonal antibody using conventional methods. Suitable antigens include enzymes (e.g., horse radish peroxidase, alkaline phosphatase, and a-galactosidase) and non-enzymatic polypeptides (e.g., serum proteins, such as BSA and globulins, and milk proteins, such as caseins).

15

**Epitopes, antigens and immunogens.**

In another aspect, the invention provides a peptide or polypeptide comprising an epitope-bearing portion of a polypeptide of the  
20 invention. The epitope of this polypeptide portion is an immunogenic or antigenic epitope of a polypeptide of the invention. An "immunogenic epitope" is defined as a part of a protein that elicits an antibody response when the whole protein is the immunogen. These immunogenic epitopes are believed to be confined to a few loci on the molecule. On the other hand, a region of a protein molecule to which  
25 an antibody can bind is defined as an "antigenic epitope." The number of immunogenic epitopes of a protein generally is less than the number of antigenic epitopes. See, for instance, Geysen, H. M. et al., Proc. Natl. Acad. Sci. USA 81:3998-4002 (1984).

As to the selection of peptides or polypeptides bearing an  
30 antigenic epitope (i.e., that contain a region of a protein molecule to which an antibody can bind), it is well known in that art that relatively short synthetic peptides that mimic part of a protein sequence are routinely capable of eliciting an antiserum that reacts with the partially mimicked protein. See, for instance, Sutcliffe, J. G. et al., Science 219:660-666 (1984). Peptides capable of eliciting  
35 protein-reactive sera are frequently represented in the primary sequence of a protein, can be characterized by a set of simple chemical rules, and are confined

neither to immunodominant regions of intact proteins (i.e., immunogenic epitopes) nor to the amino or carboxyl terminals. Peptides that are extremely hydrophobic and those of six or fewer residues generally are ineffective at inducing antibodies that bind to the mimicked protein; longer, soluble peptides, especially those  
5 containing proline residues, usually are effective. Sutcliffe et al., supra, at 661. For instance, 18 of 20 peptides designed according to these guidelines, containing 8-39 residues covering 75% of the sequence of the influenza virus hemagglutinin HA1 polypeptide chain, induced antibodies that reacted with the HA1 protein or intact virus; and 12/12 peptides from the MuLV polymerase and 18/18 from the  
10 rabies glycoprotein induced antibodies that precipitated the respective proteins.

Antigenic epitope-bearing peptides and polypeptides of the invention are therefore useful to raise antibodies, including monoclonal antibodies, that bind specifically to a polypeptide of the invention. Thus, a high proportion of hybridomas obtained by fusion of spleen cells from donors immunized with an  
15 antigen epitope-bearing peptide generally secrete antibody reactive with the native protein. Sutcliffe et al., supra, at 663. The antibodies raised by antigenic epitope bearing peptides or polypeptides are useful to detect the mimicked protein, and antibodies to different peptides may be used for tracking the fate of various regions of a protein precursor which undergoes posttranslation  
20 processing. The peptides and anti-peptide antibodies may be used in a variety of qualitative or quantitative assays for the mimicked protein, for instance in competition assays since it has been shown that even short peptides (e.g., about 9 amino acids) can bind and displace the larger peptides in immunoprecipitation assays. See, for instance, Wilson, I.A. et al., Cell 37:767-778 at 777 (1984). The  
25 anti-peptide antibodies of the invention also are useful for purification of the mimicked protein, for instance, by adsorption chromatography using methods well known in the art.

Antigenic epitope-bearing peptides and polypeptides of the invention designed according to the above guidelines preferably contain a  
30 sequence of at least seven, more preferably at least nine and most preferably between about 15 to about 30 amino acids contained within the amino acid sequence of a polypeptide of the invention. However, peptides or polypeptides comprising a larger portion of an amino acid sequence of a polypeptide of the invention, containing about 30 to about 50 amino acids, or any length up to and  
35 including the entire amino acid sequence of a polypeptide of the invention, also are considered epitope-bearing peptides or polypeptides of the invention and also

are useful for inducing antibodies that react with the mimicked protein. Preferably, the amino acid sequence of the epitope-bearing peptide is selected to provide substantial solubility in aqueous solvents (i.e., the sequence includes relatively hydrophilic residues and highly hydrophobic sequences are preferably avoided);  
5 and sequences containing proline residues are particularly preferred.

The epitope-bearing peptides and polypeptides of the invention may be produced by any conventional means for making peptides or polypeptides including recombinant means using nucleic acid molecules of the invention. For instance, a short epitope-bearing amino acid sequence may be fused to a larger  
10 polypeptide which acts as a carrier during recombinant production and purification, as well as during immunization to produce anti-peptide antibodies.

Epitope-bearing peptides also may be synthesized using known methods of chemical synthesis. For instance, Houghten has described a simple method for synthesis of large numbers of peptides, such as 10-20 mg of 248  
15 different 13 residue peptides representing single amino acid variants of a segment of the HAI polypeptide which were prepared and characterized (by ELISA-type binding studies) in less than four weeks. Houghten, R. A., Proc. Natl. Acad. Sci. USA 82:5131-5135 (1985). This "Simultaneous Multiple Peptide Synthesis (SMPS)" process is further described in U.S. Patent No. 4,631,211 to Houghten et  
20 al. (1986). In this procedure the individual resins for the solid-phase synthesis of various peptides are contained in separate solvent-permeable packets, enabling the optimal use of the many identical repetitive steps involved in solid-phase methods.

A completely manual procedure allows 500-1000 or more  
25 syntheses to be conducted simultaneously. Houghten et al., supra, at 5134.

Epitope-bearing peptides and polypeptides of the invention are used to induce antibodies according to methods well known in the art. See, for instance, Sutcliffe et al., supra; Wilson et al., supra; Chow, M. et al., Proc. Natl. Acad. Sci. USA 82:910-914; and Bittle, F.J. et al., J. Gen. Virol. 66:2347-2354  
30 (1985).

Generally, animals may be immunized with free peptide; however, anti-peptide antibody titer may be boosted by coupling of the peptide to a macromolecular carrier, such as keyhole limpet hemocyanin (KLH) or tetanus toxoid. For instance, peptides containing cysteine may be coupled to carrier using  
35 a linker such as maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), while other peptides may be coupled to carrier using a more general linking agent such as

glutaraldehyde.

Animals such as rabbits, rats and mice are immunized with either free or carriercoupled peptides, for instance, by intraperitoneal and/or intradermal injection of emulsions containing about 100 ug peptide or carrier protein and Freund's adjuvant. Several booster injections may be needed, for instance, at intervals of about two weeks, to provide a useful titer of anti-peptide antibody which can be detected, for example, by ELISA assay using free peptide adsorbed to a solid surface. The titer of anti-peptide antibodies in serum from an immunized animal may be increased by selection of anti-peptide antibodies, for instance, by adsorption to the peptide on a solid support and elution of the selected antibodies according to methods well known in the art.

Immunogenic epitope-bearing peptides of the invention, i.e., those parts of a protein that elicit an antibody response when the whole protein is the immunogen, are identified according to methods known in the art. For instance, Geysen et al., 1984, supra, discloses a procedure for rapid concurrent synthesis on solid supports of hundreds of peptides of sufficient purity to react in an enzyme-linked immunosorbent assay. Interaction of synthesized peptides with antibodies is then easily detected without removing them from the support. In this manner a peptide bearing an immunogenic epitope of a desired protein may be identified routinely by one of ordinary skill in the art. For instance, the immunologically important epitope in the coat protein of foot-and-mouth disease virus was located by Geysen et al. with a resolution of seven amino acids by synthesis of an overlapping set of all 208 possible hexapeptides covering the entire 213 amino acid sequence of the protein. Then, a complete replacement set of peptides in which all 20 amino acids were substituted in turn at every position within the epitope were synthesized, and the particular amino acids conferring specificity for the reaction with antibody were determined. Thus, peptide analogs of the epitope-bearing peptides of the invention can be made routinely by this method. U.S. Patent No. 4,708,781 to Geysen (1987) further describes this method of identifying a peptide bearing an immunogenic epitope of a desired protein.

Further still, U.S. Patent No. 5,194,392 to Geysen (1990) describes a general method of detecting or determining the sequence of monomers (amino acids or other compounds) which is a topological equivalent of the epitope (i.e., a "mimotope") which is complementary to a particular paratope (antigen binding site) of an antibody of interest. More generally, U.S. Patent No.

4,433,092 to

Geysen (1989) describes a method of detecting or determining a sequence of monomers which is a topographical equivalent of a ligand which is complementary to the ligand binding site of a particular receptor of interest. Similarly, U.S. Patent  
5 No. 5,480,971 to Houghten, R. A. et al. (1996) on Peralkylated Oligopeptide Mixtures discloses linear C1-C7-alkyl peralkylated oligopeptides and sets and libraries of such peptides, as well as methods for using such oligopeptide sets and libraries for determining the sequence of a peralkylated oligopeptide that preferentially binds to an acceptor molecule of interest. Thus, non-peptide analogs  
10 of the epitope-bearing peptides of the invention also can be made routinely by these methods.

### **Use of PEC60 pectinases in industrial processes**

15 Pectins and pectinases play an important role in the food industry. Pectinases are used in the production of fruit and vegetable juices and purees, and in the extraction of useful components, like aroma compounds, or even pharmaceuticals, from plant materials.

One of the main application of pectinases is in the production of  
20 clear fruit and vegetable juice. The production of juices from fruit and vegetables typically involves, after an optional washing step, grinding, crushing or otherwise destroying the integrity of the fruit or vegetables thus obtaining a fruit or vegetable pulp. Subsequently, the pulp may be treated with enzymes to decrease soluble pectin, a process generally referred to as maceration. After maceration, the pulp is  
25 ready for pressing, leading to a juice fraction and a residue fraction, the latter being referred to as the pomace. The juice obtained after pressing is usually pasteurised, optionally with recovery of the aroma, which may then be added back at the end of the process. In order to obtain a clear concentrate, the pasteurised juice is enzymatically depectinized with the aid of pectinases, optionally pre-  
30 concentrated, filtrated, optionally ultra-filtrated and concentrated to obtain a clear concentrate or juice which is ready for shipping and/or blending to obtain a clear apple juice for the consumer market

PEC60 pectinases are useful in all production processes in which the integrity of the cell wall should be destroyed in order to release  
35 materials from the inside of the cells, or from the cell walls.

Pectinases can also be used to destroy the connection between cells without affecting the integrity of the separate cells. When cells should remain



intact, e.g. in the preparation of potato puree PEC60 pectinases can be used to release the structural connection between the cells.

PEC60 pectinases are also able to decrease the water-binding and hence the viscosity of pectin-containing slurries, e.g. fruit or vegetable pulps, which improves the pressability/processability of the pulp.

PEC60 pectinases are able to clarify cloudy juices. Attacking the outer pectin layer of the cloud particles changes the charge of the particles, thus promoting the aggregation of the particles.

PEC60 pectinases may also be used in the retting of flax (to release the cellulose fibres from the plant tissue) and the scouring of cotton (to increase the water absorbance and dye uptake of the cotton fibres).

Plant and pectin-containing materials include plant pulp, parts of plants and plant extracts. In the context of this invention an extract from a plant material is any substance which can be derived from plant material by extraction (mechanical and/or chemical), processing or by other separation techniques. The extract may be juice, nectar, base, or concentrates made thereof. The plant material may comprise or be derived from vegetables, e.g., carrots, celery, onions, legumes or leguminous plants (soy, soybean, peas) or fruit, e.g., pome or seed fruit (apples, pears, quince etc.), grapes, tomatoes, citrus (orange, lemon, lime, mandarin), melons, prunes, cherries, black currants, redcurrants, raspberries, strawberries, cranberries, pineapple and other tropical fruits, trees and parts thereof (e.g. pollen, from pine trees).

Fruit juice that needs to be filtrated after or during processing tends to clot the filters after some time. This is due to the inability of certain enzymes to properly digest fruit polysaccharides which causes the fouling of the filters.. Use of the enzymes according to the invention prevents fouling of filters because of the unique specificity of the PEC60 pectinase.

PEC60 pectinases may conveniently be produced in microorganisms. Microbial pectinases are available from a variety of sources; Bacillus spec. are a common source of bacterial enzymes, whereas fungal enzymes are commonly produced in Aspergillus spec.

In the above processes, it is advantageous to use pectinases that are obtained by recombinant DNA techniques. Such recombinant enzymes have a number of advantages over their traditionally purified counterparts. Recombinant enzymes may be produced at a low cost price, high yield, free from contaminating agents like bacteria or viruses but also free from bacterial toxins or

contaminating other enzyme activities.

The polypeptides of the invention may be used to treat plant material including plant pulp and plant extracts. For example, they may be used to treat apple pulp and/or raw juice during the production of apple juice. They may also be used to treat liquid or solid foodstuffs or edible foodstuff ingredients. Conveniently the polypeptide of the invention is combined with suitable (solid or liquid) carriers or diluents including buffers to produce a composition or enzyme preparation. The polypeptide is typically stably formulated either in liquid or dry form. Typically, the product is made as a composition which will optionally include, for example, a stabilising buffer and/or preservative. The compositions may also include other enzymes capable of digesting plant material or pectin, for example other pectinases such as an endo-arabinanase, rhamnogalacturonases, and/or polygalacturonase. For certain applications, immobilization of the enzyme on a solid matrix or incorporation on or into solid carrier particles may be preferred. The composition may also include a variety of other plant material-degrading enzymes, for example cellulases and other pectinases.

The polypeptides and compositions of the invention may therefore be used in a method of processing plant material to degrade or modify the pectin constituents of the cell walls of the plant material

Typically, the polypeptides of the invention are used as a composition/ enzyme preparation as described above. The composition will generally be added to plant pulp obtainable by, for example mechanical processing such as crushing or milling plant material. Incubation of the composition with the plant will typically be carried out for a time of from 10 minutes to 5 hours, such as 30 minutes to 2 hours, preferably for about 1 hour. The processing temperature is preferably 10-55°C, e.g. from 15 to 25°C, optimally about 20°C and one can use 10-300g, preferably 30-70g, optimally about 50g of enzyme per ton of material to be treated. All the enzyme(s) or their compositions used may be added sequentially or at the same time to the plant pulp. Depending on the composition of the enzyme preparation the plant material may first be macerated (e.g. to a purée) or liquefied. Using the polypeptides of the invention processing parameters such as the yield of the extraction, viscosity of the extract and/or quality of the extract can be improved.

Alternatively, or in addition to the above, a polypeptide of the invention may be added to the raw juice obtained from pressing or liquefying the

plant pulp. Treatment of the raw juice may be carried out in a similar manner to the plant pulp in respect of dosage, temperature and holding time. Again, other enzymes than pectinases may be included.

5 A composition containing a polypeptide of the invention may also be used during the preparation of fruit or vegetable purees. The end product of these processes is typically heat-treated at 85°C for a time of from 1 minute to 1 hour, under conditions to partially or fully inactivate the polypeptides of the invention.

10 Due to the highly specific action on pectins the polypeptides of the invention may also be used to prepare pectins with modified characteristics, e.g. modified gelation capacities for specific applications.

The polypeptides of the invention may be added to animal feeds rich in pectin, e.g. soy-containing food, to improve the breakdown of the plant cell wall leading to improved utilisation of the plant nutrients by the animal. The  
15 polypeptides of the invention may be added to the feed or silage if pre-soaking or wet diets are preferred. Advantageously, the polypeptides of the invention may continue to degrade pectins in the feed in vivo. Fungal derived polypeptides of the invention in particular generally have lower pH optima and are capable of releasing important nutrients in such acidic environments as the stomach of an  
20 animal. The invention thus also contemplates (e.g. animal) feeds or foodstuffs comprising one or more polypeptides of the invention.

A PEC60 pectinase may also be advantageously used during the production of milk substitutes (or replacers) from soy bean. These milk substitutes can be consumed by both humans and animals. A typical problem  
25 during the preparation of these milk substitutes is the high viscosity of the soy bean slurry, resulting in the need for an undesirable dilution of the slurry to a concentration of dry solids of 10 to 15%. An enzyme preparation containing a polypeptide of the invention can be added to, or during the processing of, the slurry, enabling processing at a higher concentration (typically 40 to 50%) dry  
30 solids. Addition of pectinases of the invention to a soy suspension as described in WO 95/29598 results in a decrease in waterbinding and a concomitant decrease in viscosity of the slurry.

The enzyme may also be used in the preparation of savoury product(s), e.g. from soy bean.

35 The invention also relates to the use of the PEC60 pectinase according to the invention in a selected number of industrial and pharmaceutical

processes. Despite the long term experience obtained with these processes, the pectinase according to the invention features a number of significant advantages over the enzymes currently used. Depending on the specific application, these advantages can include aspects like lower production costs, higher specificity  
5 towards the substrate, less antigenic, less undesirable side activities, higher yields when produced in a suitable microorganism, more suitable pH and temperature ranges, better tastes of the final product as well as food grade and kosher aspects.

An important aspect of the pectinases according to the invention  
10 is that they cover a whole range of pH and temperature optima which are ideally suited for a variety of applications. For example many large scale processes benefit from relatively high processing temperatures of 50 degrees C or higher, e.g. to control the risks of microbial infections. Several pectinases according to the invention comply with this demand but at the same time they are not that heat  
15 stable that they resist attempts to inactivate the enzyme by an additional heat treatment. The latter feature allows production routes that yield final products free of residual enzyme activity. Similarly many feed and food products have slightly acidic pH values so that pectinases with acidic or near neutral pH optima are preferred for their processing. A PEC60 pectinase according to the invention  
20 complies with this requirement as well.

The polypeptides of the invention may also be added to animal feeds rich in pectin or xylogalacturonan, to reduce anti-nutritional effects of plant pectins and consequently improve production performances and welfare of the animals, as well reducing environmental pollution.

25 It has been shown that plant pectins increase digesta viscosity, fermentation and colonisation of bacterial proliferation in the gastro intestinal tract (Langhout, D.J. (1998). The role of the intestinal flora as affected by non-starch polysaccharides in broiler chicks. PhD thesis, Agricultural University Wageningen, The Netherlands). As a consequence, several digestive physiological parameters  
30 are altered (Johnson, I.T. and Gee, J.M. (1981). Effect of gel-forming gums on the intestinal unstirred layer and sugar transport in vitro. Gut 22:398-403. Johnson, I.T. and Gee, J.M. (1986). Gastrointestinal adaption in response to solublenon-available polysaccharides in the rat. British Journal of Nutrition 55:497-505, Johnson, I.T. , Gee, J.M. and Mahoney, R.R. (1984). Effect of dietary  
35 supplements of guar gum or cellulose on intestinal cell proliferation, enzyme levels and glucose transport in the rat. British Journal of Nutrition 52:477-487) resulting

- in reduced dietary nutrient, mineral and energy utilization (Langhout, D.J. and Schutte, J.B. (1996). Nutritional implications of pectins in chicks in relation to esterification and origin of pectins. Poultry Science 75:1236-1242, Smits, C.M.H., Veldman, A., Verkade, H.J. and Beynen, A.C. (1998). The inhibitory effect of
- 5 carboxymethylcellulose with high viscosity on lipid absorption in broiler chickens coincides with reduced bile salt concentration and raised microbial numbers in the small intestine. Poultry Science 77:1534-1539). The magnitude efficiency of dietary nutrient utilization is reduced, the effectivity in production performances (e.g. efficiency of producing milk, eggs, wool and meat) declines. Moreover, at
- 10 reduced dietary nutrient utilization by the animal, excretion of and environmental pollution with e.g. nitrogenous compounds and heavy metals is increased.

- Advantageous is that these fungal derived polypeptides of the invention have generally lower pH optima and their activities are therefore (more) capable of hydrolysis in such acidic environments as the stomach of an animal.
- 15 With that, anti-nutritional properties of the plant cell wall fractions are already reduced early on in the digestive tract of the animal.

## **ABSTRACT**

The invention relates to newly identified polynucleotide sequences comprising genes that encode novel pectinases isolated from *Aspergillus niger*. The invention features the full length nucleotide sequence of the novel gene, the cDNA sequence comprising the full length coding sequence of the novel pectinase as well as the amino acid sequence of the full-length functional protein and functional equivalents thereof. The invention also relates to methods of using these enzymes in industrial processes and methods of diagnosing fungal infections. Also included in the invention are cells transformed with a polynucleotide according to the invention and cells wherein a pectinase according to the invention is genetically modified to enhance or reduce its activity and/or level of expression.

## **CLAIMS**

- 1) An isolated polynucleotide hybridisable to a polynucleotide according to SEQ ID NO:1 or SEQ ID NO: 2
- 2) An isolated polynucleotide according to claim 1 hybridisable under high stringency conditions to a polynucleotide according to SEQ ID NO:1 or SEQ ID NO: 2
- 3) An isolated polynucleotide according to claims 1 or 2 obtainable from a filamentous fungus.
- 4) An isolated polynucleotide according to claim 3 obtainable from *A. niger*.
- 5) An isolated polynucleotide encoding a polypeptide comprising an amino acid sequence according to SEQ ID NO: 3 or functional equivalents thereof.
- 6) An isolated polynucleotide encoding at least one functional domain of a polypeptide according to SEQ ID NO: 3 or functional equivalents thereof.
- 7) An isolated polynucleotide comprising a nucleotide sequence according to SEQ ID NO: 1 or SEQ ID NO: 2 or functional equivalents thereof
- 8) An isolated polynucleotide according to SEQ ID NO: 1 or SEQ ID NO: 2.
- 9) A vector comprising a polynucleotide sequence according to claims 1 to 8.
- 10) A vector according to claim 9 wherein said polynucleotide sequence according to claims 1 to 8 is operatively linked with regulatory sequences suitable for expression of said polynucleotide sequence in a suitable host cell.
- 11) A vector according to claim 10 wherein said suitable host cell is a filamentous fungus
- 12) A method for manufacturing a polynucleotide according to claims 1 – 8 or a vector according to claims 9 to 11 comprising the steps of culturing a host cell transformed with said polynucleotide or said vector and isolating said polynucleotide or said vector from said host cell.
- 13) An isolated polypeptide according to SEQ ID NO: 3 or functional equivalents thereof.
- 14) An isolated polypeptide according to claim 13 obtainable from *Aspergillus niger*
- 15) An isolated polypeptide obtainable by expressing a polynucleotide according to claims 1 to 8 or a vector according to claims 9 to 11 in an appropriate host cell, e.g. *Aspergillus niger*.
- 16) Recombinant pectinase comprising a functional domain of a PEC60 polypeptide.

- 17) A method for manufacturing a polypeptide according to claims 13 to 16 comprising the steps of transforming a suitable host cell with an isolated polynucleotide according to claims 1 to 8 or a vector according to claims 9 to 11, culturing said cell under conditions allowing expression of said polynucleotide and optionally purifying the encoded polypeptide from said cell or culture medium.
- 18) A recombinant host cell comprising a polynucleotide according to claims 1 to 8 or a vector according to claims 9 to 11.
- 19) A recombinant host cell expressing a polypeptide according to claims 13 to 16.
- 20) Purified antibodies reactive with a polypeptide according to claims 13 to 16.
- 21) Fusion protein comprising a polypeptide sequence according to claims 13 to 16.



# SEQUENCE LISTING

<110> DSM NV.

<120> Novel pectinase PEC60 and uses thereof

<130> 20819EP/P0

<160> 3

<170> XML to WIPO ST.25 Converter - <http://www.biomax.de/>

<210> 1

<211> 4348

<212> DNA

<213> *Aspergillus niger*

<400> 1

ggttaaaata	ttccacggat	tatcataatg	ottgataggt	ctcaactaca	gcgaatcaaa	60
tcttatatat	gtttgggacg	cgacctctga	attcacctcc	atagtgctaa	gttggaagtg	120
accccaaggt	cctcattcat	ttaggcacta	agaagcctag	ctggctctcg	ttgagaacca	180
actttttatac	ctagccgata	gggctagacg	tcgcgtatgt	gtgtcatctc	agcttattgg	240
ccttcataat	gatacactga	tacttcccag	atagttgaat	cgcgcacttt	ttgtacctaa	300
attcgccgat	tctcagtcca	acctccaagg	attctcatca	atgagaaatc	cctatctctt	360
tttctcaaca	aaagaagctt	tcccgcagcc	gacatgccct	cccgtcggac	ccacgtctgt	420
ggcaaaatth	gaacgcagta	gcgttattgc	agtgggtctt	tccatagggc	tgaggcctct	480
aactcatcga	acagagagac	atgcagatgc	actatagttg	ccgcataccc	gataaccact	540
actatgtcca	cgggttggtg	tgggctcaag	ctttttcaaa	ccagggtttt	gcgtaagcct	600
taagagccct	ctccggcagt	ctcaaatccc	gaactcaacc	aatcggaaac	acgtttttaa	660
tggtagcctg	ggactttttt	ggacactata	tcttccatat	atcgccgagc	ttgtctgcac	720
ccttgccctgc	gtcctattaa	aatgcgggct	actgaggccg	ccagcaccgt	tcattcccca	780
ccggtctctc	attcgattcc	tccaccaatg	agtatgcccg	ccttgagtct	cctcggatcc	840
catttaactc	atatttcgaa	taaaatagat	taatgcgtgg	agaataccag	cgagcttagg	900
ggtacgctaa	gatgcgcaga	cctgggttct	cacggtttta	gctccgttcc	cggtctggtc	960
gccggcaacg	gccatgactg	ccattttccc	attcacgcca	cccctgcttg	ccctctggca	1020
gattatagat	gcctgcatgg	gcggccctct	tacacgtgct	cctgtctgaa	tttctgccag	1080
actgaactct	ccaccagct	ctttcgccat	attcgttcat	cttctgctgg	cggcgctgcc	1140
gattctatcc	catgcgcgc	tcacctatcg	cggggcggac	atttcttctc	ttttgagcta	1200
gaagatgaag	gttatagcta	caagaacctt	aaaggtcaat	cccaagcact	cgagactatc	1260
ctcgctgaag	ccggcatcaa	ttcggttcgc	cagcgtgtgt	gggtcaatt	ccagtcattg	1320
cattttacaat	ttggattaca	acttgagact	ggcaagcggg	tcaaggcggc	tggtatgagt	1380
atctatctgg	accttcatct	gagtgatata	tgggcggatc	ctagcgatta	ggtgagttga	1440
ttccgttcga	gagataatag	tcaagtcaag	aagaagccta	agctgaatta	tccggggtaa	1500
tagacaaccc	ttcctgatgg	cccaccaccg	atttcagcac	tctgaagtgg	caactataca	1560
actacactgt	tgaggtttgc	aacacgtttg	cagataacaa	cattgatatt	gagatcatct	1620
caatcggtaa	tgagatccgc	gccggctctc	tctggcctct	tggtgagacg	agcagctact	1680
caaacatcgg	tgactgctg	tactcgggcg	cttggggagt	caaggactcc	aatttagcaa	1740
cactacccaa	gattataatc	catctggatg	acggctggag	ctgggatcag	cagagttact	1800
tttataagac	ggttctgtct	acgggcgagc	tactgaacac	agacttcgac	tacttcggcg	1860
ttttataacta	cctgtttctac	agcgcacatg	taaatatagc	atctctgaag	accagcctgg	1920
cgaatctgca	gtcgatctac	aacaagcctg	tagtagtggt	ggagatgaac	tggccagttc	1980
cgtgcccaaa	tcttgagtac	tcttgtttct	cagatccgag	attgatttct	ttctcggctc	2040
cgggccagca	gcagttcttc	gagaaaactg	cagctgtggt	cgaggccact	accgatggtc	2100
aggggggtgta	ctactgggag	ccggcctgga	tcaccaatgc	tgggggtggg	tcgagctgcg	2160
atgaaaacct	tatggtggat	aggtccacgg	atgaggttta	tgcgagcttt	gatattctcg	2220
ggaagctttg	acactctgta	gaatggaatt	ttactgaggg	cgtagatatt	aatgcgtgga	2280

caactagcaa	aggcctagtt	gctactttct	tactcattga	atgattagat	tgaataatac	2340
tgtgtcaaaa	tagaagcaat	aactaatgat	athtagtaat	ccggtttccg	gagacgctcg	2400
ctacattacc	cagtctcaat	aatcaaaaga	tgtgctaact	acaattacac	agaaatatgc	2460
caggacaagt	gaagtttact	ttcaatacta	cttaagcaaa	gaaataacct	aaaggataaa	2520
tagcacctta	atctcagaaa	aaagccacgt	aagagctata	atagcactaa	ttagcctagt	2580
cttcttttagc	gttttccagg	ctatatatttc	aatcgaggtt	aactactggt	aaacagatta	2640
cacggactaa	gcacatacaa	ttaacttggt	atattttgac	ttcgattcag	cctgggtgacg	2700
gtgaaagatt	tgattgaccc	agcgatcgct	gataaacgtg	tgttttttat	gttttctaac	2760
atcaaatttg	aggatagaag	gaactctgca	agatattata	ctttcacgac	tataagtgga	2820
tagactcgtg	aatctaattc	aagtatcctg	cgcttcgaca	ctaactcatt	attgttaaat	2880
agctttctta	aatttgcctt	tatttccagt	cgaggtttga	ctcctttcaa	ccgtcccttt	2940
agagtgttta	tcttgacagc	aaatgctgca	gcgcacgact	gcttggaggg	aagttgacct	3000
cgatctgagg	cctctcaggc	aagtttttagt	ctcctatatg	tagaaaggcc	ctccatacgc	3060
ctgattagcc	acattcttaa	attaattgag	caaagttgaa	tatggcgtgt	tgtggagggtt	3120
tgtagctgtg	agggctgcgcg	goggctcggt	gtccgattac	attaacaagt	ggtaacgtaa	3180
tcggacaacg	agccggcgga	caacgagccg	ccgctctgcc	tactaaaaat	gcaacaatcg	3240
attcagatta	aagtaattca	acactatata	gataaatata	tataaataaa	ttaaataattg	3300
attttatcta	tatatattgaa	ctgattatag	tataatcttt	cgtaattaat	atagtataat	3360
agctagcatt	aagtattttt	tagtatggta	gtatctactt	ctcttagtat	caaatactgc	3420
agtatacatc	tttatcgtgg	caaattaact	gtaactaaag	gcttaacaga	taagtcatag	3480
tattccatct	tagtaaaact	ctctctatat	attcaaatag	ctttattttt	gactgaatta	3540
agtaagatta	aatattgtat	atcttggaga	tataaggcgg	tgaggggtcca	caccgacggc	3600
tcgttgtgc	gacgcttcgt	tgtccggtta	cgttacttgc	ttctctggac	taaactaccg	3660
aaggacttga	cagaagcggc	ttagcgtact	ttggatgaag	tgtccggggac	aagttataga	3720
ccataaacat	ttgcgctatt	ttttccaaac	agctgtaatg	cgtatatcga	tgtgaatatc	3780
tagaaaaatag	tcaactgcgat	agactgggtgc	ggtgaaagaa	aaggtcagct	agcagccctg	3840
gacctgattg	tcctcgcatt	aaatagtaca	cagcaaattc	accatgcata	tttgtattga	3900
atcttggggg	ctatataaaa	gcggtcatct	acagtacttc	ctcgattctc	tttctcttct	3960
tttcctttcc	atctctcatg	tcccaaaagc	agcagcggtc	atcagcccag	atggcccaac	4020
atcgataaat	tattccattg	gaagattata	ttagtcttta	gatcttttaa	taatattatt	4080
taaaatcaat	tgccctcaacc	aggatcaact	actaatgtga	tacatcaaga	gatgattgac	4140
gtgccatatt	acaaattttc	aaatgaocgat	attcgtgagc	gaagacttac	atgcctcttg	4200
tgtcatcatt	tccaaaagca	tggcccacaa	atggcgaccc	cagtttttagg	ctgagcatct	4260
tttcttgtca	agctcttctt	atcaagagtt	ctgtatgaat	atgtcttctt	cgttgcctga	4320
aatcaggacc	gcgtaccctg	agctcaga				4348

<210> 2

<211> 1191

<212> DNA

<213> *Aspergillus niger*

<220>

<221> CDS

<222> (1)..(1191)

<400> 2

atg	ccg	gct	act	gag	gcc	gcc	agc	acc	gtt	cat	tcc	cca	ccg	gtc	tct	48
Met	Pro	Ala	Thr	Glu	Ala	Ala	Ser	Thr	Val	His	Ser	Pro	Pro	Val	Ser	
1			5						10					15		
cat	tcg	att	cct	cca	cca	atg	aac	ctg	gtt	ott	cac	ggt	ttt	agc	tcc	96
His	Ser	Ile	Pro	Pro	Pro	Met	Asn	Leu	Val	Leu	His	Gly	Phe	Ser	Ser	
			20				25					30				
gtt	ccc	ggg	ctg	gtc	gcc	ggc	aac	ggc	cat	gac	tgc	cat	ttt	ccc	att	144
Val	Pro	Gly	Leu	Val	Ala	Gly	Asn	Gly	His	Asp	Cys	His	Phe	Pro	Ile	
		35					40				45					
cac	gcc	acc	cct	gct	tgc	cct	ctg	gca	gat	tat	aga	tgc	ctg	cat	ggg	192

His	Ala	Thr	Pro	Ala	Cys	Pro	Leu	Ala	Asp	Tyr	Arg	Cys	Leu	His	Gly		
50						55					60						
cgg	cct	tct	tac	acg	tgc	tcc	tgt	ctg	aat	ttc	tgc	cag	act	gaa	ctc	240	
Arg	Pro	Ser	Tyr	Thr	Cys	Ser	Cys	Leu	Asn	Phe	Cys	Gln	Thr	Glu	Leu		
65					70					75					80		
tcc	acc	cag	ctc	ttt	cgc	cat	att	cgt	tca	tct	tct	gct	ggc	ggc	gct	288	
Ser	Thr	Gln	Leu	Phe	Arg	His	Ile	Arg	Ser	Ser	Ser	Ala	Gly	Gly	Ala		
				85					90					95			
gcc	gat	tct	atc	cca	tgc	cgc	gct	cac	cta	tcg	cgg	ggc	gga	cat	ttc	336	
Ala	Asp	Ser	Ile	Pro	Cys	Arg	Ala	His	Leu	Ser	Arg	Gly	Gly	His	Phe		
			100					105					110				
ttc	tct	ttt	gag	cta	gaa	gat	gaa	ggg	tat	agc	tac	aag	aac	ctt	aaa	384	
Phe	Ser	Phe	Glu	Leu	Glu	Asp	Glu	Gly	Tyr	Ser	Tyr	Lys	Asn	Leu	Lys		
		115					120					125					
ggg	caa	tcc	caa	gca	ctc	gag	act	atc	ctc	gct	gaa	gcc	ggc	atc	aat	432	
Gly	Gln	Ser	Gln	Ala	Leu	Glu	Thr	Ile	Leu	Ala	Glu	Ala	Gly	Ile	Asn		
		130				135					140						
tcc	gtt	cgc	cag	cgt	gtg	tgg	ggg	caa	ttc	cag	tca	tgg	cat	tta	caa	480	
Ser	Val	Arg	Gln	Arg	Val	Trp	Gly	Gln	Phe	Gln	Ser	Trp	His	Leu	Gln		
				150					155					160			
ttt	gga	tta	caa	ctt	gga	gct	ggc	aag	cgg	gtc	aag	gcg	gct	ggg	atg	528	
Phe	Gly	Leu	Gln	Leu	Gly	Ala	Gly	Lys	Arg	Val	Lys	Ala	Ala	Gly	Met		
				165					170					175			
agt	atc	tat	ctg	gac	ctt	cat	ctg	aat	aac	aac	att	gat	att	gag	atc	576	
Ser	Ile	Tyr	Leu	Asp	Leu	His	Leu	Asn	Asn	Asn	Ile	Asp	Ile	Glu	Ile		
			180					185					190				
atc	tca	atc	ggg	aat	gag	atc	cgc	gcc	ggg	ctt	ctc	tgg	cct	ctt	ggg	624	
Ile	Ser	Ile	Gly	Asn	Glu	Ile	Arg	Ala	Gly	Leu	Leu	Trp	Pro	Leu	Gly		
			195				200					205					
gag	acg	agc	agc	tac	tca	aac	atc	ggg	gca	ctg	ctg	tac	tcg	ggc	gct	672	
Glu	Thr	Ser	Ser	Tyr	Ser	Asn	Ile	Gly	Ala	Leu	Leu	Tyr	Ser	Gly	Ala		
		210				215					220						
tgg	gga	gtc	aag	gac	tcc	aat	tta	gca	aca	cta	ccc	aag	att	ata	atc	720	
Trp	Gly	Val	Lys	Asp	Ser	Asn	Leu	Ala	Thr	Leu	Pro	Lys	Ile	Ile	Ile		
				230					235					240			
cat	ctg	gat	gac	ggc	tgg	agc	tgg	gat	cag	cag	agt	tac	ttt	tat	aag	768	
His	Leu	Asp	Asp	Gly	Trp	Ser	Trp	Asp	Gln	Gln	Ser	Tyr	Phe	Tyr	Lys		
				245				250						255			
acg	gtt	ctg	tct	acg	ggc	gag	cta	ctg	aac	aca	gac	ttc	gac	tac	ttc	816	
Thr	Val	Leu	Ser	Thr	Gly	Glu	Leu	Leu	Asn	Thr	Asp	Phe	Asp	Tyr	Phe		
			260					265					270				
ggc	gtt	tta	tac	tac	ctg	ttc	tac	agc	gca	tca	gta	aat	ata	gca	tct	864	
Gly	Val	Leu	Tyr	Tyr	Leu	Phe	Tyr	Ser	Ala	Ser	Val	Asn	Ile	Ala	Ser		
		275					280					285					
ctg	aag	acc	agc	ctg	gcg	aat	ctg	cag	tcg	atc	tac	aac	aag	cct	gta	912	
Leu	Lys	Thr	Ser	Leu	Ala	Asn	Leu	Gln	Ser	Ile	Tyr	Asn	Lys	Pro	Val		
		290				295					300						
gta	gtg	gtg	gag	atg	aac	tgg	cca	gtt	ccg	tgc	cca	aat	cct	gag	tac	960	
Val	Val	Val	Glu	Met	Asn	Trp	Pro	Val	Pro	Cys	Pro	Asn	Pro	Glu	Tyr		
				310					315					320			
tct	tgt	ttc	tca	gat	ccg	aga	ttg	att	tct	ttc	tcg	gtc	gcg	ggc	cag	1008	
Ser	Cys	Phe	Ser	Asp	Pro	Arg	Leu	Ile	Ser	Phe	Ser	Val	Ala	Gly	Gln		
				325					330					335			
cag	cag	ttc	ctc	gag	aaa	ctg	gca	gct	gtg	gtc	gag	gcc	act	acc	gat	1056	
Gln	Gln	Phe	Leu	Glu	Lys	Leu	Ala	Ala	Val	Val	Glu	Ala	Thr	Thr	Asp		
			340				345					350					
ggg	cag	ggg	gtg	tac	tac	tgg	gag	ccg	gcc	tgg	atc	acc	aat	gct	ggg	1104	

Gly	Gln	Gly	Val	Tyr	Tyr	Trp	Glu	Pro	Ala	Trp	Ile	Thr	Asn	Ala	Gly		
		355					360					365					
gtg	ggg	tcg	agc	tgc	gat	gaa	aac	ctt	atg	gtg	gat	agg	tcc	acg	gat		1152
Val	Gly	Ser	Ser	Cys	Asp	Glu	Asn	Leu	Met	Val	Asp	Arg	Ser	Thr	Asp		
		370				375					380						
gag	gtt	tat	gcg	agc	ttt	gat	att	ctc	ggg	aag	ctt	tga					1191
Glu	Val	Tyr	Ala	Ser	Phe	Asp	Ile	Leu	Gly	Lys	Leu						
385					390					395							

<210> 3  
 <211> 396  
 <212> PRT  
 <213> Aspergillus niger

<400> 3

Met	Pro	Ala	Thr	Glu	Ala	Ala	Ser	Thr	Val	His	Ser	Pro	Pro	Val	Ser		
1				5					10					15			
His	Ser	Ile	Pro	Pro	Pro	Met	Asn	Leu	Val	Leu	His	Gly	Phe	Ser	Ser		
			20					25					30				
Val	Pro	Gly	Leu	Val	Ala	Gly	Asn	Gly	His	Asp	Cys	His	Phe	Pro	Ile		
		35				40						45					
His	Ala	Thr	Pro	Ala	Cys	Pro	Leu	Ala	Asp	Tyr	Arg	Cys	Leu	His	Gly		
	50				55						60						
Arg	Pro	Ser	Tyr	Thr	Cys	Ser	Cys	Leu	Asn	Phe	Cys	Gln	Thr	Glu	Leu		
65					70					75					80		
Ser	Thr	Gln	Leu	Phe	Arg	His	Ile	Arg	Ser	Ser	Ser	Ala	Gly	Gly	Ala		
				85				90						95			
Ala	Asp	Ser	Ile	Pro	Cys	Arg	Ala	His	Leu	Ser	Arg	Gly	Gly	His	Phe		
			100				105						110				
Phe	Ser	Phe	Glu	Leu	Glu	Asp	Glu	Gly	Tyr	Ser	Tyr	Lys	Asn	Leu	Lys		
		115				120						125					
Gly	Gln	Ser	Gln	Ala	Leu	Glu	Thr	Ile	Leu	Ala	Glu	Ala	Gly	Ile	Asn		
	130					135					140						
Ser	Val	Arg	Gln	Arg	Val	Trp	Gly	Gln	Phe	Gln	Ser	Trp	His	Leu	Gln		
145					150					155					160		
Phe	Gly	Leu	Gln	Leu	Gly	Ala	Gly	Lys	Arg	Val	Lys	Ala	Ala	Gly	Met		
				165				170						175			
Ser	Ile	Tyr	Leu	Asp	Leu	His	Leu	Asn	Asn	Asn	Ile	Asp	Ile	Glu	Ile		
			180					185					190				
Ile	Ser	Ile	Gly	Asn	Glu	Ile	Arg	Ala	Gly	Leu	Leu	Trp	Pro	Leu	Gly		
		195					200					205					
Glu	Thr	Ser	Ser	Tyr	Ser	Asn	Ile	Gly	Ala	Leu	Leu	Tyr	Ser	Gly	Ala		
	210					215					220						
Trp	Gly	Val	Lys	Asp	Ser	Asn	Leu	Ala	Thr	Leu	Pro	Lys	Ile	Ile	Ile		
225					230					235					240		
His	Leu	Asp	Asp	Gly	Trp	Ser	Trp	Asp	Gln	Gln	Ser	Tyr	Phe	Tyr	Lys		
				245				250						255			
Thr	Val	Leu	Ser	Thr	Gly	Glu	Leu	Leu	Asn	Thr	Asp	Phe	Asp	Tyr	Phe		
			260				265					270					
Gly	Val	Leu	Tyr	Tyr	Leu	Phe	Tyr	Ser	Ala	Ser	Val	Asn	Ile	Ala	Ser		
		275				280						285					
Leu	Lys	Thr	Ser	Leu	Ala	Asn	Leu	Gln	Ser	Ile	Tyr	Asn	Lys	Pro	Val		
	290					295					300						
Val	Val	Val	Glu	Met	Asn	Trp	Pro	Val	Pro	Cys	Pro	Asn	Pro	Glu	Tyr		
305					310					315				320			
Ser	Cys	Phe	Ser	Asp	Pro	Arg	Leu	Ile	Ser	Phe	Ser	Val	Ala	Gly	Gln		

				325					330					335			
Gln	Gln	Phe	Leu	Glu	Lys	Leu	Ala	Ala	Val	Val	Glu	Ala	Thr	Thr	Asp		
			340					345						350			
Gly	Gln	Gly	Val	Tyr	Tyr	Trp	Glu	Pro	Ala	Trp	Ile	Thr	Asn	Ala	Gly		
		355					360					365					
Val	Gly	Ser	Ser	Cys	Asp	Glu	Asn	Leu	Met	Val	Asp	Arg	Ser	Thr	Asp		
	370					375					380						
Glu	Val	Tyr	Ala	Ser	Phe	Asp	Ile	Leu	Gly	Lys	Leu						
385					390					395							

**PCT/EP2003/005726**

